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Inhibition of Lung Metastasis by Synthetic and Recombinant Fragments of Human Fibronectin with Functional Domains

Ikuro Saiki,^{1,3} Jun Murata,¹ Takashi Makabe,¹ Yoshihiro Matsumoto,¹ Yoichi Ohdate,² Yasutoshi Kawase,² Yuki Taguchi,² Tomoko Shimojo,² Fusao Kimizuka,² Ikuoshin Kato,² and Ichiro Azuma¹

¹Institute of Immunological Science, Hokkaido University, Kita-15, Nishi-7, Kita-ku, Sapporo 060 and

²Biotechnology Research Laboratories, Takara Shuzo Co. Ltd., Seta 3-4-1, Otsu 520-01

We have investigated the antimetastatic effect of synthetic or recombinant peptides containing the functional domains of fibronectin on experimental and spontaneous lung metastases of murine tumor cells. CS1 peptide which is present within type III homology connecting segment (IIICS) as well as C-274 (cell-binding domain) were able to inhibit experimental lung metastasis when co-injected intravenously (iv) with B16-BL6 melanoma cells, while H-271 (heparin-binding domain) could not. In the spontaneous metastasis model, multiple iv administrations of CS1 or C-274 after surgical excision of primary tumors caused a significant reduction of metastatic colonies in the lung. Both CS1 and C-274 significantly inhibited cell adhesion and migration to fibronectin-coated substrates when added freely in solution. CS1 peptide also inhibited the cell adhesion and migration to laminin-coated substrates, but C-274 did not. H-271 did not have any inhibitory effect on cell adhesion or migration to either of the substrates. Similarly, CS1 inhibited tumor invasion to both Matrigel/fibronectin- and Matrigel/laminin-coated filters, whereas C-274 inhibited the invasion to only Matrigel/fibronectin-coated filter. These results indicate that CS1 peptide of fibronectin, lacking the Arg-Gly-Asp-containing domain, actively inhibits tumor metastases in spontaneous and experimental metastasis models. The use of such a peptide might offer a promising therapeutic approach for combatting or preventing cancer metastasis.

Key words: Recombinant fibronectin fragment Metastasis Haptotactic migration Cell adhesion Invasion

During the sequential steps of metastasis, metastasizing tumor cells interact with various host cells (platelets, lymphocytes or endothelial cells) and/or extracellular matrix and basement membrane components (fibronectin and laminin).¹⁻⁴⁾ This encounter may lead to enhancement of the survival, arrest, or invasiveness of tumor cells.^{2,4-7)} Such specific interaction is therefore a fundamental event in the metastatic process.

DNA technology has resulted in the identification of the primary structures of some cell adhesion proteins such as fibronectin,⁸⁾ vitronectin⁹⁾ and laminin,^{10,11)} and the receptors for some adhesive molecules on the cell surface. A common and characteristic Arg-Gly-Asp (RGD) core sequence in cell-binding domain of fibronectin and other related adhesion molecules has been shown to contribute to the cell functions including adhesion, spreading and migration of cells.¹²⁻¹⁴⁾ Several studies have suggested that some synthetic peptides based on the adhesion molecules that are present in cell matrices, basement membranes, or plasma can modulate the mechanism involved in the metastasizing function of tumor cells. A proteolytic fragment of laminin has been used to inhibit experimental metastasis of murine melanoma and it has been shown to bind to a high-affinity 70 kDa glycoprotein receptor on the surface of cells.^{15,16)} Humphries *et al.*^{17,18)} have shown that treatment of tumor cells *ex vivo* with GRGDS peptide, which is present in the cell-binding domain of fibronectin, was able to inhibit experimental metastasis of a murine melanoma. We have recently reported that poly(RGD), which consists of repeated RGD sequences, inhibited experimental and spontaneous lung metastases of tumor cells, as well as cell-adhesive properties, more effectively than RGD-containing oligopeptides.¹⁹⁻²¹⁾ On the other hand, McCarthy *et al.*¹⁶⁾ have shown that the *ex vivo* pre-treatment of tumor cells with a purified 33-kDa heparin-binding fragment of fibronectin, which promotes tumor cell adhesion by an RGD-independent mechanism,²²⁾ effectively inhibited experimental pulmonary metastases of melanoma or fibrosarcoma. The CS1 peptide, which is present within a 33-kDa fibronectin fragment²²⁾ and lies between the carboxyl-terminal heparin- and fibrin-binding domains, has been shown to promote cell adhesion and spreading,^{23,25)} but its inhibitory effect on tumor metastasis has not previously been examined.

In the present study, we examined the effect of synthetic and recombinant human fibronectin fragments containing functional domains on the lung metastases of

³ To whom correspondence should be addressed.

murine melanoma cells, and studied their biological characteristics in relation to the metastatic cascade in order to gain insight into the action of the polypeptides.

MATERIALS AND METHODS

Recombinant fibronectin polypeptides and other reagents
 We prepared two kinds of recombinant fibronectin fragment (C-274 and H-271) by expressing human fibronectin cDNA in *E. coli*, using an expression vector pUC118N/119N described by Maki *et al.*²⁶ C-274 and H-271 correspond to the cell- and heparin-binding domains of fibronectin, respectively (Fig. 1). Two plasmids, pLF5 and pLF2435, were used as a source of the cDNA.²⁷ The cell-binding polypeptide C-274 was expressed through a recombinant plasmid pTF7221 which had been constructed mainly from pLF5 and pUC119N. The plasmid pTF7221 was derived from pTF7121, which expresses a cell-binding polypeptide C-279, with five additional amino acids at the carboxyl-terminus of C-274. The heparin-binding polypeptide H-271 was expressed by use of a recombinant plasmid pHD101; this had been constructed from pLF2435 and pUC118N. Detailed accounts for these constructions and expressions will be presented elsewhere (Kimizuka *et al.*, manuscript in preparation). The recombinant fragment C-274 expressed in *E. coli* was purified from the cell extract by DEAE ion exchange chromatography, followed by gel filtration. H-271 was purified by CM ion exchange chromatography, followed by affinity chromatography using heparin as a ligand. The polypeptides thus prepared were analyzed on a 15% SDS-polyacryl-

amide gel under reducing conditions and visualized by CBB staining to verify their purity. CS1 peptide (DELQLVTLPHPNLHGPEILDVPST) which is present within type III connecting segment (IIICS) of human fibronectin,^{23,24} was synthesized at Takara Shuzo Co., Ltd. using an LKB Biolynx 4170 peptide synthesizer with solid-phase Fmoc chemistry. The peptide was purified by preparative reverse-phase HPLC on a C-18 column with a gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid. The amino-terminal sequence was checked with an automated peptide sequencer 477A (Applied Biosystems Inc., Foster City, CA). These polypeptides were dissolved in Ca^{2+} - and Mg^{2+} -free phosphate-buffered saline (PBS) before use. Purified human fibronectin was purchased from Biomedical Technologies Inc., MA. Basement membrane Matrigel (containing laminin, collagen type IV, heparan sulfate proteoglycan and entactin) and rabbit anti mouse laminin IgG were obtained from Collaborative Research Inc., MA. All the reagents and media in this study were endotoxin-free (< 0.1 ng/ml), as determined by a colorimetric assay (Pyrodisk, Seikagaku Kogyo Co. Ltd., Tokyo).

Animals Specific pathogen-free mice of C57BL/6 strain, 8–13 weeks old, were purchased from Shizuoka Laboratory Animal Center, Hamamatsu. Mice were maintained in the Laboratory of Animal Experiment, Institute of Immunological Science, Hokkaido University, under laminar air-flow conditions. All mice used in this study were sex-matched.

Cells Highly metastatic B16-BL6 melanoma cells, obtained by an *in vitro* selection procedure for invasion,²⁸ were kindly provided by Dr. I. J. Fidler, M.D. Anderson

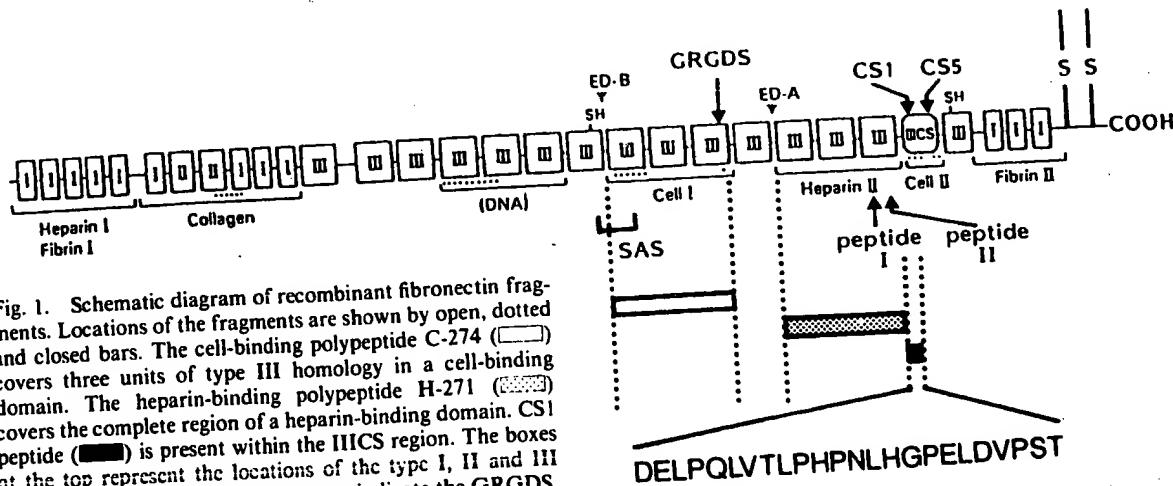


Fig. 1. Schematic diagram of recombinant fibronectin fragments. Locations of the fragments are shown by open, dotted and closed bars. The cell-binding polypeptide C-274 (□) covers three units of type III homology in a cell-binding domain. The heparin-binding polypeptide H-271 (▨) covers the complete region of a heparin-binding domain. CS1 peptide (■) is present within the IIICS region. The boxes at the top represent the locations of the type I, II and III homology repeats. The vertical arrows indicate the GRGDS, CS1, CS5,^{23–25} and peptide I and II sites.²⁶ ED-A and ED-B indicate that extra domains arise from alternative splicing, respectively.

Cancer Center, Houston, TX. B16-BL6 cells were maintained as monolayer cultures in Eagle's minimal essential medium (MEM) supplemented with 7.5% fetal bovine serum (FBS), vitamin solution, sodium pyruvate, non-essential amino acids and L-glutamine.

Microassay for cell adhesion The cell attachment assay was carried out by the method described.¹⁹ B16-BL6 melanoma cells in an exponential growth phase were incubated for 24 h in MEM containing 5% FBS supplemented with 0.3 μ Ci/ml [¹²⁵I]iododeoxyuridine ([¹²⁵I]IUDR) (specific activity, 200 mCi/mmol, New England Nuclear, Boston, MA). The cells were washed twice in warm PBS to remove unbound radiolabels, harvested by adding 0.02% EDTA for 1 min at 37°C, and resuspended in cold serum-free MEM to form a single-cell suspension. [¹²⁵I]IUDR-labeled tumor cells (2×10^4) in a volume of 0.05 ml/well were added to microculture wells precoated with fibronectin or laminin. The cultures were incubated at 37°C for 30 min and then the wells were washed four times with PBS to remove unattached cells. The remaining substrate-bound tumor cells were lysed with 0.1 ml of 0.1 N NaOH. The lysate was absorbed on cotton swabs and monitored for radioactivity by gamma counting. The binding capacity (No. of cells bound/substrate) was expressed as follows;

$$\text{Binding capacity} = \frac{\text{cpm of targets bound to substrate}}{\text{cpm of total tumor cells added}} \times \frac{\text{total number of tumor cells added.}}{10^4}$$

Haptotactic migration assay Tumor cell migration along a gradient of substratum-bound fibronectin or laminin was assayed in a Transwell cell culture chamber (Costar No. 3422, Cambridge, MA) according to the methods reported by McCarthy *et al.*,²⁰ with some modifications.³⁰ Polyvinylpyrrolidone-free polycarbonate filters with an 8.0 μ m pore size (Nucleopore, Pleasanton, CA) were precoated with either 5 μ g of fibronectin or laminin in a volume of 50 μ l on their lower surfaces, and dried overnight at room temperature. The coated filters were washed extensively in PBS and then dried immediately before use. Log-phase cell cultures of tumor cells were harvested with 1 mM EDTA in PBS, washed three times with serum-free MEM, and resuspended to a final concentration of 2×10^6 /ml in MEM with 0.1% bovine serum albumin (BSA). Cell suspensions (100 μ l) with or without agents were added to the upper compartment, and incubated for the appropriate number of hours at 37°C in a 5% CO₂ atmosphere. The filters were fixed with methanol, and stained with hematoxylin and eosin. The cells on the upper surface of the filters were removed by wiping them with a cotton swab. The cells that had migrated to various areas of the lower surface were manually counted under a microscope in 5 predeter-

mined fields at a magnification of 400, and each assay was performed in triplicate.

Invasion assay The invasive activity of tumor cells was assayed according to the method reported by Albini *et al.*,³¹ with some modifications.³⁰ Briefly, the lower surface of the filters was precoated with fibronectin or laminin, as described above. The Matrigel was diluted to 100 μ g/ml with cold PBS, applied to the upper surfaces of the filters (5 μ g/filter), and dried at room temperature under a hood. The filters thus prepared were designated Matrigel/fibronectin- or Matrigel/laminin-coated filters, respectively. The subsequent procedures were the same as those of the haptotactic migration assay.

Experimental and spontaneous lung metastases C57BL/6 mice were given iv injections (0.2 ml/mouse) of B16-BL6 melanoma (3×10^4) admixed with various concentrations of the recombinant fragments in PBS. Fourteen days after the inoculation of tumors, the mice were killed and the number of lung tumor colonies was recorded (experimental lung metastasis). In a spontaneous pulmonary metastasis assay, mice were given sc injections of B16-BL6 melanoma cells (5×10^5) into the right hind footpad. The primary tumor were surgically removed on day 21 after tumor inoculation. The recombinant polypeptides were administered iv on various days before or after the amputation. Mice were killed 14 days after the amputation. The lungs were fixed in Bouin's solution and the number of lung tumor colonies was counted under a dissecting microscope.

Statistical analysis The statistical significance of the differences between groups was determined by applying Student's two-tailed *t* test.

RESULTS

Inhibition of experimental lung metastasis by synthetic and recombinant domain polypeptides We investigated the effect of domain polypeptides on experimental lung metastasis caused by intravenous co-injection with B16-BL6 melanoma cells (Table I). Increasing doses of CS1 or C-274 led to significant reductions in the number of lung tumor nodules ($P < 0.001$). In contrast, H-271 achieved no reduction of lung tumor colonies at any of the doses used in this study.

Inhibition of spontaneous lung metastases by systemic administration of synthetic and recombinant polypeptides We first examined the effect on spontaneous lung metastasis by multiple systemic administrations of C-274, which contains the cell-binding RGD sequence of fibronectin (Expt. I of Table II). Seven or five intermittent iv injections of 100 μ g of C-274 before or after surgical excision of primary tumors achieved a statistically significant reduction of lung tumor colonies, while three intermittent treatment had no effect. In this exper-

Table I. Effect of Recombinant Fibronectin Fragments on Experimental Lung Metastases by Intravenous Injection of B16-BL6 Melanoma Cells

Administered iv with	Dose (μg/mouse)	No. of metastases on day 14	
		Expt. I	Expt. II
Untreated	—	45 ± 10 (32-55)	87 ± 11 (72-98)
C-274	40	30 ± 6 (26-39)	90 ± 20 (69-113)
	100	42 ± 10 (25-51)	59 ± 19 (41-85)*
	200	13 ± 16 (3-32)*	28 ± 14 (14-48)**
	500	51 ± 6 (44-55)	76 ± 13 (60-92)
	1000	32 ± 14 (19-48)	66 ± 20 (44-90)
H-271	40	50 ± 4 (29-40)	105 ± 16 (81-124)
	100	30 ± 6 (25-40)	23 ± 2 (22-25)**
	200	12 ± 6 (6-21)**	
	500	8 ± 6 (4-18)**	
	1000		
CS1	40		
	200		
	1000		

Five C57BL/6 mice per group were inoculated iv with B16-BL6 cells (3×10^6) admixed with or without recombinant fragments of fibronectin. Mice were killed 2 weeks after tumor inoculation and tumor colonies in the lungs were counted.

*; $P < 0.02$, **; $P < 0.001$

Table II. Therapeutic Effect of Synthetic and Recombinant Domain Polypeptides of Fibronectin on Spontaneous Lung Metastases by Intrafootpad Injection of B16-BL6 Melanoma Cells

Administered iv with	Dose (μg/mouse)	Primary tumor size on day 21 (mm ± SD)	No. of lung metastases on day 35	P ^{a)}
Expt. I	—	10 ± 3	65 ± 17 (41-90)	
Untreated	—	11 ± 3	21 ± 11 (13-37)	0.01
C-274	Days 7, 9, 11, 13, 15, 17, 19	100 ± 7	36 ± 10 (23-44)	0.02
	7, 10, 13, 16, 19	100 ± 5	74 ± 23 (43-93)	
	7, 13, 19	100 ± 3	21 ± 13 (8-38)	0.01
C-274	Days 22, 24, 26, 28, 30, 32, 34	100 ± 7	29 ± 2 (26-31)	0.01
	22, 25, 28, 31, 34	100 ± 5	57 ± 29 (31-98)	
	22, 28, 34	100 ± 3		
Expt. II	—	10 ± 2	86 ± 14 (70-101)	
Untreated	—	10 ± 3	44 ± 14 (23-63)	0.01
C-274	Days 22, 24, 26, 28, 30, 32, 34	50 ± 7	31 ± 15 (12-49)	0.001
		100 ± 7		
H-271	Days 22, 24, 26, 28, 30, 32, 34	100 ± 7	89 ± 24 (56-122)	
		100 ± 7		
CS1	Days 22, 24, 26, 28, 30, 32, 34	100 ± 7	28 ± 15 (4-43)	0.001

Five C57BL/6 mice per group were given fibronectin polypeptides iv on the indicated days after tumor inoculation. Primary tumors were surgically removed on day 21 and mice were killed 2 weeks after tumor excision.

a) Compared with the untreated control by Student's two-tailed *t* test.

Table III. Effect of Synthetic and Recombinant Domain Polypeptides on the Growth of B16-BL6 Melanoma Cells *in vitro*

Treatment	Concentration (μg/ml)	Incorporation of [³ H]thymidine into the cells (cpm · SD)
Untreated (medium)	—	40637 · 2629
C-274	5	43859 · 2870
	50	37689 · 8404
	500	45075 · 3578
H-271	5	41100 · 3995
	50	41886 · 3838
	500	46198 · 1249
CS1	5	38117 · 10895
	50	42030 · 2710
	500	43268 · 3426
TNF-α (2 · 10 ⁴ U/ml)		22110 · 5063

B16-BL6 cells ($5 \cdot 10^3$) were incubated with MEM medium containing 5% FBS, domain polypeptides, or recombinant TNF-α for 3 days at 37°C. The cultures were pulsed with 0.5 μCi of [³H]thymidine for the last 4 h before the termination.

iment, iv administration of the polypeptide before the amputation did not affect the primary tumor size (growth) at the time of amputation (on day 21). We next examined the effect on spontaneous lung metastasis of B16-BL6 by seven systemic administrations of the domain polypeptides. The polypeptides were administered iv into the lateral tail vein of mice after the amputation of the primary tumors. As shown in Expt. II of Table II, iv administrations of 100 μg of CS1 as well as C-274 after surgical excision of the primary tumors resulted in a reduction of lung tumor colonization. H-271, however, did not affect the inhibition of lung metastasis. We also examined the direct effect of synthetic and recombinant polypeptides on the growth of B16-BL6 melanoma cells *in vitro*. Table III shows that the incubation of tumor cells with various concentrations of polypeptides did not affect the incorporation of [³H]thymidine into tumor cells. Recombinant human tumor necrosis factor (TNF-α) as a positive control potently inhibited the cell growth *in vitro*.

Effect of the polypeptides on the invasion of tumor cells
Tumor cell invasion into extracellular matrices and basement membranes is a crucial step in the complex multi-stage process of metastasis.^{15, 32, 33} We therefore examined the effect of these antimetastatic polypeptides on tumor cell invasion of Matrigel (reconstituted basement membrane components) (Table IV). The invasion of tumor cells through the Matrigel/fibronectin-coated filters was significantly inhibited by the addition of 100 or

Table IV. Effect of Recombinant Fragments of Fibronectin on Tumor Cell Invasion to Matrigel/fibronectin- or Matrigel/laminin-coated Filters

Treatment	Dose (μg/ml)	No. of invaded cells (mean ± SD)	
		Fibronectin	Laminin
None		44 · 3	59 · 6
C-274	100	26 · 4 **	
	500	10 · 2 **	58 · 4
H-271	500	49 · 13	61 · 14
CS1	100	32 · 6 *	43 · 6 *
	500	21 · 2 **	30 · 5 **
Rabbit anti mouse laminin IgG	10		32 · 5 **

Filters were precoated with 5 μg of fibronectin or laminin on their lower surfaces, and with Matrigel (5 μg) on their upper surfaces. B16-BL6 melanoma cells ($2 \cdot 10^3$ /well) in 0.1% BSA medium were seeded with or without fragments of fibronectin into the upper compartment of a Transwell cell culture chamber. Anti mouse laminin IgG was added to the lower compartment of the chamber. After an 11-h incubation, the invaded cells on the lower surfaces were counted visually.

*: P < 0.01, **: P < 0.001

500 μg/ml of CS1 or C-274 into the upper compartment of the chamber, whereas H-271 showed no anti-invasive activity. In contrast, the invasion through the Matrigel/laminin-coated filters was significantly inhibited by CS1 peptide or rabbit anti mouse laminin IgG (10 μg/ml), but not by C-274 or H-271.

Effect of the polypeptides on tumor cell adhesion Since metastatic cells presumably interact with extracellular matrix components (cell adhesion proteins) during the invasive process,^{2, 4, 7} we tested the effect of the polypeptides on tumor cell adhesion to fibronectin- or laminin-coated wells. Fibronectin and laminin promoted the adhesion of B16-BL6 melanoma cells when they were immobilized (coated) on the culture dish surface. However, few B16-BL6 cells in serum-free MEM attached themselves to BSA-coated or uncoated plastic substrates. Figure 2 shows that C-274 at various concentrations ranging from 20 to 500 μg/ml inhibited specifically the adhesion of tumor cells to fibronectin substrate but not to the laminin substrate. H-271 was unable to inhibit tumor cell adhesion to either fibronectin or laminin. In contrast, CS1 inhibited tumor cell adhesion to both fibronectin and laminin substrates in a dose-dependent manner.

Effect of the polypeptides on the migration of tumor cells
We also investigated the effect of the domain polypeptides on the haptotactic migration of tumor cells to filters precoated on their lower surfaces with 5 μg of

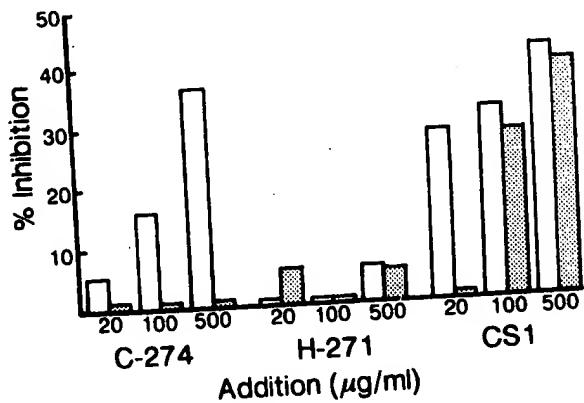


Fig. 2. Inhibition of tumor cell adhesion to fibronectin or laminin by the domain polypeptides. ^{125}I -labeled B16-BL6 cells (2×10^4) were added to wells precoated with 1 μg of fibronectin (white bar) or 1 μg of laminin (hatched bar) in the presence or absence of C-274, H-271 or CS1 peptides. After a 30-min incubation, nonadherent cells were washed away and the adherent cells were counted. Percent inhibition of cell adhesion was quantitated as: $1 - (\text{the number of cells bound in the presence of inhibitor} / \text{the number of cells bound in the absence of the inhibitor}) \times 100$.

Table V. Inhibition of Haptotactic Migration of B16-BL6 Melanoma to Fibronectin- or Laminin-coated Filters by Fibronectin Fragments

Addition	Dose ($\mu\text{g/ml}$)	No. of migrated cells \pm SD	
		Fibronectin	Laminin
None	—	53 \pm 5	97 \pm 5
C-274	20	35 \pm 6**	
	100	29 \pm 3**	
	500	22 \pm 4**	93 \pm 6
H-271	20	54 \pm 10	
	100	48 \pm 5	
	500	45 \pm 10	96 \pm 10
CS-1	20	42 \pm 4*	96 \pm 6
	100	30 \pm 4**	75 \pm 6**
	500	24 \pm 4**	60 \pm 6**
Rabbit anti mouse laminin IgG	10		30 \pm 3**

Filters in a Transwell chamber were precoated with 5 μg of fibronectin or laminin on their lower surfaces. B16-BL6 cells (2×10^3) were incubated with the domain polypeptides in the upper compartment or with anti mouse laminin IgG in the lower compartment for 4 h. The migrant cells on the lower surfaces were counted visually.

*; $P < 0.01$, **; $P < 0.001$.

fibronectin or laminin without the matrix barrier (Matrigel) on the upper surface (Table V). B16-BL6 cells were incubated for 4 h at 37°C with polypeptides in the upper compartment of a Transwell cell culture chamber. CS1 or C-274 inhibited tumor cell migration to the fibronectin-coated filter in a dose-dependent manner, whereas H-271 did not. CS1 or rabbit anti mouse laminin IgG inhibited cell migration to the laminin-coated filter, but C-274 and H-271 did not.

DISCUSSION

We have attempted to elucidate the regulatory mechanisms involved in cell functions such as adhesion and motility that are important during the metastatic process. Tumor cell adhesion to components of the extracellular matrix, in particular fibronectin, is an important aspect of several steps of the metastatic process. Previous studies have utilized cell-adhesion-promoting fragments of laminin or heparin-binding fibronectin fragments or the synthetic RGDS peptide of fibronectin to inhibit experimental metastasis of tumor cells in mice.¹⁵⁻¹⁸ Our current studies extend those earlier studies, clearly demonstrating that synthetic and recombinant polypeptides of the functional domains of fibronectin can be used to inhibit the experimental and spontaneous lung metastases of tumor cells in mice and the penetration of tumor cells through reconstituted basement membrane (Matrigel) *in vitro*.

CS1 peptide and C-274 (containing RGD sequence) were able to inhibit the experimental and spontaneous lung metastases of melanoma cells, but did not affect the primary tumor size at the time of amputation. We also observed that incubation of B16-BL6 melanoma cells with these polypeptides for 3 days did not affect the growth of tumor cells *in vitro* (Table III). Therefore, the inhibitory effect of these polypeptides on lung tumor metastasis may not be due to their direct cytotoxicity against tumor cells.

Although some studies of proteolytic fragments of laminin¹⁹ or fibronectin,¹⁶ or synthetic RGD-containing peptides of fibronectin^{17, 18} have been performed with an experimental metastasis model, it is of particular interest that, in our experiments with a spontaneous metastasis model, CS1 and C-274 showed therapeutic potential against tumor metastasis following systemic administration.

The penetration of tumor cells into basement membranes involves such distinct events as the attachment of tumor cells, the secretion by the tumor cells of enzymes that cause the degradation of the adjacent basement membrane, and the migration of the cells into the target tissue. C-274 (containing the cell-binding RGD sequence) specifically inhibited tumor cell invasion through

a Matrigel/fibronectin filter, and adhesion and migration to fibronectin substrates, but not to Matrigel/laminin or laminin substrates. H-271, however, did not have any such effects. On the other hand, CS1 peptide was able to inhibit the adhesion, migration and invasion of tumor cells to both fibronectin- and laminin-coated substrates. These results suggest that the inhibitory effects of CS1 peptide depend on interference with the interaction between tumor cells and extracellular matrix molecules by an RGD-independent mechanism, while C-274 (i.e. possibly the RGD sequences in the molecule) may inhibit specifically the adhesion, migration and invasion through interference with the RGD/integrin interaction in fibronectin-mediated cell behavior. Further detailed study will be needed to determine the precise mechanism by which CS1 inhibits tumor metastasis and invasion.

The mechanism of the inhibition of tumor metastasis by the administration of C-274 or CS1 is not well understood. Polypeptide-mediated inhibition of tumor metastasis may be related to interference with cellular adhesive interactions in the multistep metastatic process, including tumor invasion into the surrounding tissues during the intravasation and extravasation steps or at a target organ. Some possibilities include the acceleration of the release of arrested tumor cells from the lung and the inhibition of their lodgment by the polypeptides. Further study will be needed to examine the optimum administration timings of CS1 and C-271 and the detailed mechanism of the inhibitory effect.

On the other hand, H-271 polypeptide containing a heparin-binding domain II was unable to inhibit lung metastasis, although McCarthy *et al.*^{16, 22} have reported that a purified 33 kDa heparin-binding fragment of fibronectin, which includes H-271 and CS1 polypeptides, inhibited experimental metastases. The difference in anti-metastatic effects between H-271 and the proteolytic 33 kDa fragment of fibronectin may arise because the 33 kDa fragment contained a partial sequence of an IIICs insert of fibronectin, i.e. a CS1 portion that inhibited tumor metastasis as shown in Tables I and II, whereas H-271 did not contain the sequence.

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Despite the importance of RGD sequences in the cell-binding domain of fibronectin, CS1 in the IIICs insert of fibronectin deserves particular attention regarding its possible contributions to fibronectin functions. Humphries *et al.*^{23, 24} have reported that CS1 peptide contains a cell-adhesion-promoting activity for different cell types. Adhesion of T or B lymphocytes as well as melanoma cells to this region of fibronectin has been demonstrated to involve an $\alpha_4\beta_1$ integrin receptor which is RGD-independent.^{34, 35, 37} We recently observed that CS1 or H-271 inhibited liver metastasis of L5178Y-ML25 lymphoma cells in CDF1 mice (manuscript in preparation). This may imply that multiple receptors including $\alpha_4\beta_1$, $\alpha_5\beta_1$ or $\alpha_1\beta_1$ integrins interact with fibronectin, apparently at different sites in the molecule. Interestingly, our results showed that CS1 inhibited tumor cell invasion, adhesion and migration not only to fibronectin but also to laminin in an RGD-independent manner. This suggests that CS1 peptide derived from fibronectin is able to modulate the interaction between tumor cells (presumably surface receptor) and laminin as well as fibronectin. We are now examining in detail the regulatory mechanisms that allow the polypeptides to inhibit tumor metastasis and invasion.

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Inhibition of the Biological Effects of Anti-Collagen Synthetic Peptides That Inhibit Experimental Metastasis of B16-F10 Murine Melanoma Cells

Martin J. Humphries,^{1,2} Kenneth M. Yamada,¹ and Kenneth Iden^{1,2}

¹Department of Oncology, Howard University Cancer Center, Washington, District of Columbia 20060; and ²Membrane Biochemistry Section, Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Abstract

The experimental metastasis of B16-F10 murine melanoma cells is blocked by the anti-cell adhesive pentapeptide Gly-Arg-Gly-Asp-Ser (GRGDS) derived from the central cell-binding domain of fibronectin. In this report, we show that peptide treatment substantially extends the survival time for mice injected intravenously with B16-F10 cells (8/8 vs. 0/8 mice alive at 150 d), thereby demonstrating the potential efficacy of GRGDS treatment in protection against metastatic colonization. We have also examined the specificity of GRGDS activity by testing a series of related homologues for their effects on experimental metastasis. The overall profile of the relative inhibitory activities of these peptides closely matched their previously established capacity to disrupt adhesion *in vitro*. Lung retention studies with radiolabeled B16-F10 cells revealed an accelerated rate of cell loss from the lung 0–6 h after coinjection with the active peptide GRGDS. This early effect of GRGDS was consistent with its short circulatory half-life, which was found to be 8 min. Taken together, these results suggest that peptide-mediated inhibition of pulmonary colonization is due to interference with B16-F10 cell adhesion to structures in the target organ. Possible peptide interference in tumor cell–blood cell interactions was examined in order to assess (a) possible biological side-effects of peptide treatment and (b) whether such interactions might be an alternative mechanism for GRGDS-mediated inhibition of pulmonary colonization. GRGDS was found to retain full inhibitory activity when coinjected with B16-F10 cells into mice in which platelet function was impaired by acetylsalicylic acid treatment or into thrombocytopenic mice treated with antiplatelet serum (76–93% inhibition of colony formation). These data suggest that platelet involvement in the effects of the peptide is minimal. Similarly, GRGDS was also found to be a potent inhibitor of experimental metastasis in natural killer (NK) cell-deficient beige mice (86% inhibition), thereby discounting the possibility that GRGDS artificially enhanced NK cell activity. We conclude as a result of these studies that cell-binding fibronectin peptides are specific inhibitors of experimental metastasis that prolong survival, that they appear to function by blocking the adhesion of B16-F10 cells to structures in the target organ, and that they do not appear to act through side effects on certain metastasis-related blood cell functions. In the future, deriva-

tives of fibronectin peptides may be potentially useful prophylactic agents for interfering with the process of metastasis.

Introduction

The adhesive interaction of metastatic tumor cells with components of the extracellular matrix appears to be obligatory for successful target organ colonization (1–3). It is clear that attachment to, and penetration of, basement membrane and connective tissue structures takes place at multiple stages of the metastatic cascade. Recent studies have implicated the basement membrane glycoprotein laminin in metastatic colonization. Either preincubation (3, 4) or coculture (5) of murine melanoma cells with the intact laminin molecule was found to enhance metastasis, while proteolytic cell-binding fragments have the opposite effect (3, 4).

Peptides containing the sequence Arg-Gly-Asp-Ser (RGDS), originally identified in the cell-binding domain of fibronectin, have been shown to be competitive, reversible inhibitors of cellular adhesion to extracellular matrix components (6–8). A relatively strict requirement for the particular sequence of amino acids in these peptides has been established as a result of extensive analysis *in vitro* (6, 7, 9, 10). We have recently reported that Gly-Arg-Gly-Asp-Ser (GRGDS) inhibits experimental metastasis when coinjected intravenously into syngeneic mice with lung-colonizing B16-F10 murine melanoma cells (11). Some of the specificity of peptide inhibition of adhesion that had been observed previously *in vitro* was also found in experimental metastasis assays, since two control peptides containing minor sequence alterations by amino acid inversion or conservative substitution were inactive (11). This result suggested that GRGDS might function by interfering with B16-F10 cell adhesion to target organ structures. In pulmonary retention studies using radiolabeled tumor cells, GRGDS was found to promote cell loss shortly after injection (11), a result consistent with early interference in tumor cell–extracellular matrix interactions as the mechanism of action of the peptide. However, since the process of tumor metastasis is so complex (12, 13), it is conceivable that GRGDS may block at multiple sites. Recent evidence suggests that certain blood cell populations, particularly platelets and natural killer (NK)¹ cells, play pivotal roles in regulation of tumor metastasis (reviewed in references 14–17), and it is possible that GRGDS may inhibit experimental metastasis by interfering with the normal functioning of these cells.

Many different types of tumor cells are known to elicit aggregation of platelets *in vitro* (18–21) and to cause thrombocytopenia when injected into experimental animals (18, 22). Both of these properties have been correlated with the meta-

Address reprint requests to: Dr. Humphries, Howard University Cancer Center, 2041 Georgia Avenue N.W., Washington, D.C. 20060.

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1. Abbreviations used in this paper: APS, antiplatelet serum; NK, natural killer (cell).

static potential of the tumor cells. Morphologic analysis of the process of experimental organ colonization has demonstrated that blood platelets bind in a time-dependent manner to capillary-arrested tumor cells over the first 4 h postinjection (23, 24). This interaction may stabilize the initial adhesion of tumor cells to the endothelial layer by increasing the size of tumor cell emboli as well as by shielding the tumor cells from immune detection. The results of preliminary trials employing antiplatelet drugs for prevention of metastasis have been equivocal partly because of the side effects of the drugs on normal vascular function (reviewed in references 14, 15, and 25). Nevertheless, in all cases examined to date, drug-induced thrombocytopenia has proven an effective inhibitor of experimental metastasis, therefore providing solid evidence for the role of platelets in the metastatic process (26-29).

NK cells are a subpopulation of lymphoid cells that appear to have an important role in the destruction of circulating tumor cell emboli (reviewed in references 16 and 17). Studies with animals exhibiting low levels of NK activity, including cyclophosphamide- or β -estradiol-treated mice, 3-wk-old mice (30-34), and beige mice (34-37), have demonstrated an increased frequency of experimental and spontaneous metastasis. Similarly, activation of NK cell activity *in vivo* by administration of biological response modifiers renders the host more resistant to metastatic colonization (38, 39). Since NK cells can recognize and eliminate a wide variety of tumor cells without prior sensitization, it is likely that they are able to lyse targets shortly after their release into the vascular system.

As an initial step towards assessing potential prophylactic applications of GRGDS-like peptides and in order to gain insight into the mechanism of peptide action, we have tested the effect of GRGDS on survival of mice injected with B16-F10 cells. Furthermore, we have examined the specificity of peptide inhibition together with the contribution of tumor cell-blood cell interactions to GRGDS-mediated inhibition of B16-F10 experimental metastasis.

Methods

Materials. Synthetic peptides, synthesized by Peninsula Laboratories (Belmont, CA), were further purified by Sephadex G10 chromatography in 10 mM NH_4HCO_3 as described (11). Peptides were > 98% pure by reversed-phase HPLC. [^{35}S]sodium sulfate (25-40 Ci/mg) was obtained from Amersham Corp. (Arlington Heights, IL). Acetylsalicylic acid was from Sigma Chemical Co. (St. Louis, MO).

Cell culture and experimental metastasis assay. B16-F10 murine melanoma cells, a line selected *in vivo* for high pulmonary colonization (40), were cultured as described (41, 42). Cultures were routinely tested for microbial infection and were verified to be mycoplasma-free. Cells were detached for 2 min with 0.02% EDTA and resuspended carefully to $5.0-7.5 \times 10^5/\text{ml}$ in Dulbecco's modified Eagle's minimal essential medium (DME; Gibco, Grand Island, NY). Synthetic peptides, dissolved in divalent cation-free Dulbecco's phosphate-buffered saline (PBS⁻; Gibco), were neutralized and sterilized by microfiltration and mixed with cells. 0.2-ml aliquots containing the specified amount of peptide together with $5.0-7.5 \times 10^4$ cells were injected slowly into the lateral tail vein of groups of eight C57BL/6 or C57BL/6^{+/+} mice (Charles River Breeding Laboratories, Wilmington, MA or Jackson Labs., Bar Harbor, ME, respectively) as described (11, 43). The animals were killed 14 d later with ether, their lungs were excised and fixed in 10% formaldehyde, and surface melanoma colonies were counted.

Pulmonary retention of radiolabeled B16-F10 cells. B16-F10 cells were plated at $2 \times 10^6/75\text{-cm}^2$ flask and the next day were labeled with 5-[^{125}I]iododeoxyuridine (0.4 $\mu\text{Ci}/\text{ml}$, 200 mCi/mmol; New England

Nuclear, Boston, MA) for 18 h in growth medium (44). Cells were then detached with EDTA as above, and aliquots were injected with or without GRGDS into the lateral tail vein of C57BL/6 mice. At different times, groups of three mice were killed by cervical dislocation, their lungs were excised, and then the radiolabel present in the DNA of dead cells was removed by extensive washing with 70% ethanol. Pulmonary radioactivity was then determined with a gamma counter (44).

Preparation of antiplatelet serum. Rabbit anti-mouse platelet serum (APS) was prepared exactly as described previously (27). Mouse platelets were prepared by differential centrifugation of whole mouse blood (Pel-Freez, Rogers, AR). 30-ml of blood was centrifuged in polypropylene tubes at 225 g for 10 min at room temperature, and the supernatant solution was resedimented to remove red blood cells. The resulting platelet-rich plasma was centrifuged at 1,400 g for 15 min at room temperature to sediment platelets. The pellet was washed and resuspended to a density of 2×10^9 cells/ml with 0.9% NaCl, and was stored on ice prior to intravenous injection of 10^9 platelets into adult New Zealand white rabbits. Platelets were counted as follows: 5 μl of platelet-rich plasma was mixed with 45 μl of cerebrospinal fluid diluting fluid (0.2% crystal violet in 10% glacial acetic acid; AJP Scientific, Clifton, NJ) and incubated at room temperature for 10 min to lyse red blood cells. The solution was then diluted appropriately (~10-fold), and the platelets were counted by hemocytometer. A boost injection of platelets prepared in the same way was administered after 20 d, and the animals were exsanguinated 14 d later. Antisera were absorbed sequentially for 1 h at room temperature with mouse plasma protein-Sepharose affinity gel and packed mouse red blood cells before decomplementation at 56°C for 30 min.

Tritium labeling of synthetic peptides. Peptides were labeled with [^3H]sodium borohydride by reductive methylation as described by Tack et al. (45). 12 mg of peptide was dissolved in 250 μl PBS⁻, adjusted to pH 9 and transferred to a glass Reacti-vial (Kontes Glass, Vineland, NJ). 200 μl 2.5 Ci/ml [^3H]sodium borohydride in 0.01 M NaOH (74 Ci/mmol; New England Nuclear) and 180 μl 0.4 M formaldehyde were added, and the solution was incubated at room temperature for 10 min. Unreacted [^3H]sodium borohydride was removed by Sephadex G10 chromatography in 10 mM NH_4HCO_3 , and the void volume peak containing ^3H -peptide was pooled and lyophilized. The labeled peptides were further purified by chromatography in 10 mM NH_4HCO_3 on a 1.6 \times 90 cm Fractogel TSK HW-40(S) column (Pierce Chemical Co., Rockford, IL) and lyophilized.

Clearance of peptides from the circulation. C57BL/6 mice were injected as described above with 0.2-ml aliquots of PBS⁻ containing either 6 μmol ^3H -peptides (1 $\mu\text{Ci}/\text{ml}$) or [^{35}S]sodium sulfate (10 $\mu\text{Ci}/\text{ml}$). After various times, 5- μl samples of blood, withdrawn by cardiac puncture from groups of three mice, were added to 20 μl of 3% sodium citrate in glass scintillation vials. 0.2 ml of solubilizer consisting of Protosol (New England Nuclear):ethanol (1:2 [vol/vol]) was added, and the vials were incubated at 54°C for 2 h. After cooling, 0.12 ml 30% hydrogen peroxide was added slowly, and the vials were incubated a further 30 min at 54°C. After cooling, 6 ml of Biofluor (New England Nuclear) and 0.2 ml of 0.5 M HCl were added before scintillation counting.

Results

Effect of GRGDS treatment on survival of mice. In an earlier study (11), we reported that a single injection of 3 mg of the pentapeptide GRGDS along with B16-F10 murine melanoma cells was sufficient to reduce the pulmonary colonization potential of these cells by > 90%. The inhibition was specific, nontoxic, and did not result from an impairment of cellular tumorigenicity. Furthermore, a 12-mo follow-up of the consequences of peptide administration in the absence of tumor cells in the growth rate of mice revealed no adverse effects. Therefore, in order to assess the possible protection afforded to

C57BL/6 mice by GRGDS treatment, we examined the effect of a single coinjection of 3 mg of peptide on the survival time of animals receiving i.v.-injected B16-F10 cells.

As shown in Fig. 1, all mice receiving 3×10^4 B16-F10 cells alone died between 32 and 46 d after injection (mean survival time of 35.8 d). Autopsy revealed the presence of extensive melanotic lesions in the lungs. Mice receiving GRGDS, however, were all long-term survivors (still alive at 150 d postinjection; Fig. 1). Similar results were obtained after injection of 1×10^4 B16-F10 cells (0/8 survivors if untreated and 8/8 peptide-treated mice alive after 150 d postinjection; data not shown). This finding suggests that, in combination with normal host defenses, peptide treatment of the mice completely prevented the establishment of metastatic colonies and thereby essentially cured the animals. When this experiment was repeated with a larger inoculum of melanoma cells that would not be expected to completely inhibit colonization (5×10^4 cells; see reference 11 for a discussion of the dependence of GRGDS inhibition on inoculum size), no significant difference in survival time was observed (mean survival of 28.9 ± 1.1 and 27.3 ± 1.9 days for control and peptide-treated animals, respectively). Since the prognosis for mice bearing even a small number of melanotic colonies is very poor, it is likely that complete inhibition of colonization is required to observe prolongation of survival. From the results in Fig. 1, it is clear that by choosing an appropriate inoculum size, GRGDS can completely inhibit establishment of metastases, and that this inhibition translates into prolonged survival. We conclude as a result of these experiments that if an appropriate means of administration of GRGDS can be found, the peptide can prevent experimental metastatic disease such that host survival time is greatly enhanced.

Specificity of peptide inhibition of experimental metastasis. In order to examine in detail the specificity of inhibition of B16-F10 experimental metastasis by fibronectin-derived synthetic peptides, we have tested a larger library of homologous tetra- and pentapeptide molecules. These peptides include two that are found in the primary structure of the central cell-interaction site of fibronectin (RGDS and GRGDS), a truncated version of the latter peptide that lacks the COOH-terminal serine residue (GRGD), an inverted peptide with reversed sequence (SDGR), and a homologue of GRGDS that contains a conservative substitution of glutamic acid for aspartic acid (GRGES).

Consistent with our previous report (11), coinjection of 6 μmol (3 mg) GRGDS with 5×10^4 B16-F10 cells resulted in highly significant inhibition of melanotic colony formation (90% inhibition, $P = 0.002$, Table I). Assuming an extracellular

Table I. Effect of Different Fibronectin Peptide Homologues on B16-F10 Experimental Metastasis

Treatment	Colony number (mean \pm SE)	Inhibition %	Colony number, median (and range)	<i>P</i>
Control	83.2 \pm 30.1	—		65 (23–164)
GRGDS	8.5 \pm 5.5	90	2 (0–44)	0.002
RGDS	17.3 \pm 2.6	79	18 (8–25)	0.01
GRGES	82.0 \pm 24.6	1	71 (26–174)	NS
GRGD	59.2 \pm 12.8	29	41 (37–100)	NS
SDGR	33.0 \pm 7.3	60	36 (12–53)	0.05

Each peptide (6 μmol) was coinjected with 5×10^4 B16-F10 cells into C57BL/6 mice as described in Methods. Melanotic colony formation was determined 14 d later. Statistical significance was estimated using the Mann-Whitney U test.

lar fluid volume in the mouse of 6 ml, the initial concentration of GRGDS would be 1 mM. Since the affinities of fibronectin and GRGDS for the fibronectin receptor have previously been determined by direct binding studies to be 0.8 and 100 μM , respectively (46), injection of 6 μmol peptide is reasonable for achieving competitive inhibition of a fibronectin-related adhesive event. GRGES displayed little or no activity (Table I), demonstrating the extreme specificity of GRGDS inhibition. The shorter peptide, RGDS, was also highly active in blocking pulmonary colonization (79% inhibition at 6 μmol , $P = 0.01$, Table I), although this degree of inhibition was less than that obtained with GRGDS. These results support a role for the RGDS tetrapeptide recognition signal in the colonization process, and rule out the possibility that GRGDS-mediated inhibition was an artifact of that particular pentapeptide amino acid sequence. At present, RGDS appears to be the minimal sequence that retains high inhibitory activity in cellular adhesion assays (6, 7, 9, 10). It is notable, however, that in studies in which RGDS and GRGDS have been compared directly for their ability to inhibit cell adhesion to fibronectin, the presence of the NH₂-terminal glycine residue appeared to significantly increase the specific activity of the peptide (6, 10). Such findings in vitro are consistent with the in vivo results presented in Table I.

Peptides lacking the COOH-terminal serine residue of the active RGDS tetrapeptide appear to have greatly reduced ability to disrupt cellular adhesion to extracellular matrix molecules in vitro, although a significant effect has been observed at concentrations $> 500 \mu\text{g/ml}$ (10 and unpublished data). This result suggests that although the COOH-terminal residue may not be absolutely required for peptide function, it does contribute quite significantly to peptide activity in vitro. Coinjection of 6 μmol GRGD with B16-F10 cells resulted in 29% inhibition of colony formation (Table I). Since the initial blood level of GRGD is likely to be $\sim 1 \text{ mg/ml}$, assuming a blood volume of $\sim 2 \text{ ml}$, this low level of inhibition is consistent with the similarly minimal effects obtained in vitro.

Recently, it has been reported that the tetrapeptide SDGR, an inversion of the authentic RGDS sequence, also contains substantial inhibitory activity in cell adhesion assays (10). The reason for this somewhat surprising discovery is currently unknown, but it has coincidentally led to the identification of an

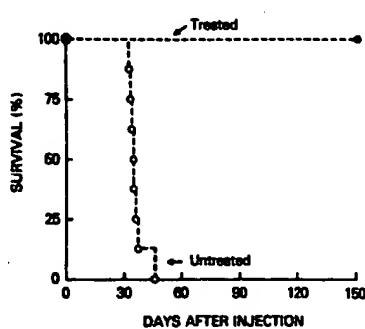


Figure 1. Effect of a single administration of 3 mg of GRGDS on survival of C57BL/6 mice injected with 3×10^4 B16-F10 cells. Melanoma cells and peptide were coinjected into the lateral tail vein and animal survival was then monitored as a function of time.

apparent difference in the adhesive responses of two different cell types. Whereas RGDS and SDGR were almost equally active in blocking fibroblast spreading on immobilized fibronectin (10), the reverse peptide was without activity in assays measuring platelet binding and adhesion to fibronectin (47). From the data presented in Table I, it is clear that SDGR is also inhibitory in the experimental metastasis assay (60% inhibition at 6 μ mol, $P = 0.05$). Similar data have been obtained in a number of independent experiments at similar or higher significance levels. Combining the results of three experiments, the inhibition by SDGR was 54%, a value significant at $P < 0.001$. As discussed further below, this result provides indirect evidence against the involvement of platelets in peptide-mediated inhibition of colonization.

Circulatory retention of GRGDS. In our initial report (11), we performed one experiment to investigate the time frame over which GRGDS promoted cell loss from the lung. Using an assay designed to monitor retention of radiolabeled B16-F10 cells in the target organ, it was found that coinjection of GRGDS had two apparent effects on the kinetics of cell loss. First, there was a slight but reproducible drop in the number of cells that initially arrested in the lung, and second, over the initial postinjection period the number of arrested cells declined progressively until after 6 h, fivefold fewer cells were present in the lungs of peptide-treated mice (11, and see also Fig. 3). To extend these findings, we have performed studies to examine the behavior of GRGDS in the circulation and to test whether the peptide is only active immediately after injection; i.e., is its principal mode of action to block the initial arrest of B16-F10 cells, or is it able to induce cell loss subsequent to tumor cell arrest?

One obviously important question concerns the actual half-life of GRGDS in the blood of mice. GRGDS was labeled with [³H]sodium borohydride by reductive methylation and injected into the lateral tail vein of C57BL/6 mice. At various times, blood samples were withdrawn by cardiac puncture and the level of radioactivity determined. The results shown in Fig. 2 demonstrate that the clearance of [³H]GRGDS was both rapid and biphasic. A very rapid initial decrease in circulating peptide level (to ~ 25% of the initial level) was detected from 0 to 2 min after injection, assuming a blood volume of 2 ml.

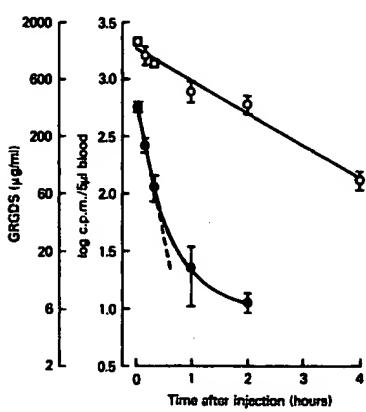


Figure 2. Clearance of [³H]GRGDS and [³⁵S]sulfate from the vascular system of C57BL/6 mice. The level of radioactivity in blood samples was determined as described in Methods and used to calculate the estimated blood concentration of peptide. The first samples were withdrawn after 2 min. The deviation from linearity of the GRGDS curve is due to the number of counts

approaching background. The half-life of each substance was calculated by regression analysis. Each point represents the mean of a group of three mice \pm SE. (○) Sulfate; (●) GRGDS.

This initial decrease was probably due to rapid equilibration with interstitial fluid, since parallel injections of [³⁵S]sulfate exhibited a similar rapid decline (Fig. 2). The sulfate ion has been used to estimate interstitial fluid volume since it is distributed without obstruction into the extracellular space, yet its renal clearance occurs only at a relatively slow rate (48, 49). Control experiments demonstrated that GRGDS mock-labeled with sodium borohydride was equally effective as the unlabeled peptide in blocking cellular adhesion to fibronectin, thereby demonstrating that the labeling procedure did not affect the biological activity of GRGDS.

After initial equilibration (> 2 min postinjection), [³H]-GRGDS and [³⁵S]sulfate were cleared at different rates (Fig. 2). The respective half-times were 8 and 63 min. The clearance rate of GRGDS is therefore similar to that of other small protein molecules tested in previous studies (50, 51). The rapid clearance of GRGDS suggests that peptide-mediated inhibition of experimental metastasis occurs over the initial postinjection time period, since the concentration of peptide in plasma would be expected to fall to < 100 μ g/ml within 30 min of injection. In order to exclude the possibility that the difference in antimetastatic activity of the peptide homologues employed in Table I might be inversely related to their half-life in the circulation, the clearance rate of [³H]-GRGES was determined. This was found to be 9 min, a value very similar to that of GRGDS.

In view of the rapid clearance rate of GRGDS, we next investigated whether the effects of the peptide *in vivo* might be restricted to inhibition of tumor cell attachment rather than to dislodgement subsequent to arrest. It was conceivable that the decrease in the percentage of initially arrested cells induced by GRGDS (11) might be sufficient to trigger the rapid loss of remaining cells by host defense mechanisms. We have tested this hypothesis in the experiment shown in Fig. 3. Different numbers of [¹²⁵I]iododeoxyuridine-labeled B16-F10 cells ($3-5 \times 10^4$) were injected in the absence of GRGDS, and the time course of pulmonary retention was followed for 6 h. The rates of cell loss were compared with that of 5×10^4 cells coinjected with 6 μ mol GRGDS. We predicted that if the sole action of GRGDS was to reduce the number of initially attached cells, then the time course of cell loss in the presence of the peptide would follow that of an appropriate untreated inoculum designed to supply the same number of initially arrested cells. From the results in Fig. 3, however, GRGDS also appears to act subsequent to initial arrest, since cell inocula that yield fewer attached cells initially ($3-4 \times 10^4$ cells injected) were subsequently lost at a slower rate than peptide-treated cells that initially arrested at higher levels. We conclude, therefore, that despite its short circulatory half-life, GRGDS is able to promote B16-F10 cell dislodgement over the initial postinjection time period.

Tumor cell-blood cell interactions in GRGDS-mediated inhibition of experimental metastasis: role of platelets. Although the most likely mechanism of action of GRGDS in blocking experimental metastasis is direct interference with the adhesion of B16-F10 cells to extracellular matrix molecules in the blood vessel wall, the complexity of the metastatic process suggests that there are many other possible cell functions that, if blocked by peptide, could result in decreased colonization. The most obvious alternative site of action of GRGDS would be effects on blood cells. It is now well established that both platelets (13, 15) and NK cells (16, 17) play prominent roles in

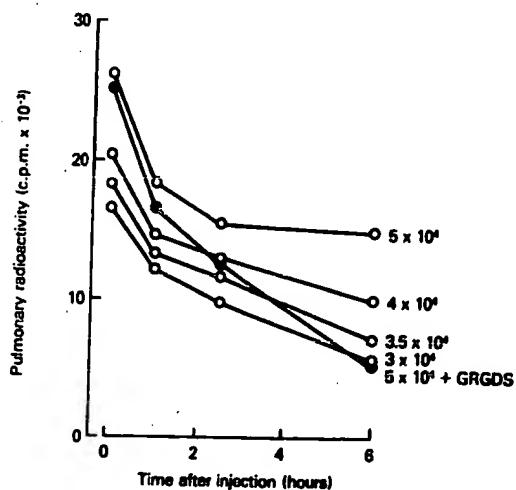


Figure 3. Effect of GRGDS on pulmonary retention of B16-F10 cells. Cultures were labeled with [¹²⁵I]iododeoxyuridine and a range of initial cell numbers (indicated at the right) was injected into C57BL/6 mice with or without 6 μ mol GRGDS. At the indicated times after injection, pulmonary radioactivity was determined as described in Methods. Each point represents the mean of three mice. For the sake of clarity error bars are omitted, but in virtually every case experimental points differed from the mean by $\leq 10\%$. (○) Control; (●) GRGDS.

the metastatic cascade, although with opposing effects. It is therefore possible that GRGDS could either inhibit platelet function or stimulate NK cell activity. These two systems are the most likely to be affected, since they both act soon after tumor cell release into the circulation. Platelets are thought to promote metastasis by binding to tumor cells in the blood and consequently facilitating lodgement in target organs. This process is postulated to occur shortly after tumor cell release. Alternatively, a potential artifactual stimulation of NK cell function by GRGDS is possible because NK activity requires no prior stimulation, unlike classical immune system function.

As mentioned above, our finding that the reverse peptide SDGR is active in blocking experimental metastasis of B16-F10 cells (Table I) suggests that platelets may not be the

site of action of GRGDS, since SDGR is known to lack inhibitory activity in assays of platelet adhesion (47). However, since these assays could be quite different from situations encountered *in vivo*, we have carried out two experiments to test the effect of platelet-tumor cell aggregation on GRGDS-mediated inhibition of pulmonary colonization.

First, the circulating platelet count in mouse blood was decreased by injection of a specific antiplatelet serum (APS), and then the effect of GRGDS on B16-F10 colonization was examined. Injection of 0.05 ml of APS reduced the platelet count by 55 \pm 19% 24 h later. Injection of an equal volume of preimmune serum produced no detectable reduction in platelet number. As anticipated from the results of Gasic et al. (27), the decrease in platelet count induced by APS resulted in a dramatic reduction in the ability of untreated B16-F10 cells to colonize the lungs of C57BL/6 mice (73% inhibition, $P = 0.005$, Table II). In other experiments, this level of inhibition was found to be close to maximal. Injection of preimmune serum gave insignificant inhibition (9.3%; Table II). This result confirms the key role that platelets play in promotion of experimental metastasis. When control mice, and mice preinjected 24 h earlier with 0.05 ml APS or preimmune serum, were challenged with B16-F10 cells in the presence or absence of 6 μ mol GRGDS, the peptide was able to inhibit colonization in all animals to a similar degree (76%, 77%, and 79% inhibition in control, APS-treated and preimmune serum-treated animals, respectively; Table II). Mice treated with both APS and GRGDS exhibited 94% inhibition of B16-F10 colonization relative to untreated animals (Table II), demonstrating an additivity of action. Since GRGDS is still fully active when the contribution of platelet adhesion is removed from the system, we conclude that its mechanism of action is to a large degree unrelated to inhibition of platelet function.

As a second test, mice were given acetylsalicylic acid in their drinking water for 3 d prior to challenge with B16-F10 cells in the presence or absence of GRGDS. It has been reported previously that administration of acetylsalicylic acid reduces platelet reactivity to collagen and also reduces experimental metastasis, although the extent of inhibition obtained was not as great as with APS (18, 27, 29, 52). Systemic administration of acetylsalicylic acid (3.5 mM in drinking water) to C57BL/6 mice reduced the level of B16-F10 cell pulmonary

Table II. Effect of APS on GRGDS-mediated Inhibition of B16-F10 Experimental Metastasis

Treatment of mice	Treatment of cells	Colony number (mean \pm SE)	Inhibition %	Colony number, median (and range)	P
None	Control	92.2 \pm 26.4	—	73 (33-187)	—
None	GRGDS	22.1 \pm 5.5	76	19 (6-42)	0.05
0.05 ml PI	Control	83.6 \pm 13.4	9	79 (55-134)	NS
0.05 ml PI	GRGDS	17.8 \pm 3.6	81	16 (11-35)	0.002
0.05 ml APS	Control	25.3 \pm 5.8	73	24 (11-48)	0.005
0.05 ml APS	GRGDS	5.9 \pm 1.9	94	9 (0-10)	<0.001

6×10^4 B16-F10 cells were coinjected with or without 6 μ mol GRGDS into groups of control C57BL/6 mice or mice preinjected 24 h earlier with 0.05 ml preimmune (PI) rabbit serum or APS. Colony formation was determined after 14 d. P values were estimated by Mann-Whitney U test. Each GRGDS-treated group was compared with its respective untreated control and the PI and APS controls were compared with the non-antibody-treated control.

colonization by 28% ($P = 0.05$, Table III). This result is similar to that reported previously (18, 52). Coinjection of GRGDS with B16-F10 cells into acetylsalicylic acid-treated mice resulted in nearly equal inhibition of colonization as when tumor cells were injected into untreated mice (90% and 93%, respectively, $P < 0.001$, Table III), thereby confirming the results obtained with APS and reinforcing the notion that platelets are not involved in GRGDS-mediated inhibition of experimental metastasis.

In addition to assays modulating platelet number or function by addition of exogenous agents, we also performed a control to test the possibility that GRGDS itself might affect the platelet count. However, 1 h after injection of either 6 μmol GRGDS or an equivalent volume of PBS⁻, there was no change in the number of platelets in C57BL/6 mouse blood ($3.02 \pm 0.73 \times 10^9/\text{ml}$ and $2.77 \pm 0.74 \times 10^9/\text{ml}$, respectively, compared with $3.07 \pm 0.33 \times 10^9/\text{ml}$ for uninjected mice).

Tumor cell-blood cell interactions in GRGDS-mediated inhibition of experimental metastasis: role of NK cells. As discussed above, the early effect of GRGDS on tumor cell dislodgement from the target organ implies that it is unlikely that any long-term stimulation of the immune system is involved in peptide-mediated inhibition of colonization. However, in order to rule out this possible mechanism, GRGDS was injected into mice 2 d prior to challenge with B16-F10 cells. From the data presented earlier in Fig. 2, it is clear that this preinjection time should be more than adequate to allow clearance of GRGDS from the circulation. If the peptide were acting via long-term immune stimulation, then the stimulus would have already been given to the mice and colonization could still have been inhibited. The number of colonies induced by 5×10^4 B16-F10 cells in control mice and in mice preinjected with 6 μmol GRGDS were similar (29.6 ± 5.2 and 33.5 ± 3.6 , respectively), whereas mice in which GRGDS and B16-F10 cells were coinjected showed significant inhibition of colonization (7.4 ± 2.2 colonies, 75% inhibition, $P < 0.001$).

If the immune system were to make a significant contribution to GRGDS-mediated inhibition of experimental metastasis, it would be more likely to involve components that do not require prior sensitization. In view of the proposed role of NK cells in reducing tumor metastasis, we have examined their role in peptide inhibition of B16-F10 experimental metastasis. In order to test directly the involvement of NK cells, we have employed C57BL/6 mice bearing the beige mutation. These mice are characterized by a specific genotypic defect in

NK activity (35, 36). If the sole action of GRGDS were to stimulate NK function, the peptide would be expected to be inactive in blocking colonization in beige mice. From the results presented in Table IV, it is clear that for the same number of B16-F10 cells injected, many more colonies were produced in beige mice. This result is consistent with previous reports (37, 53) and illustrates the role that NK cells play in removal of metastatic cells from the circulation. Interestingly, GRGDS was equally potent in blocking experimental metastasis of B16-F10 cells in both C57BL/6 and C57BL/6^{beige} mice (86% and 81% inhibition, respectively, $P < 0.001$ for both, Table IV), providing strong evidence that NK cells play an insignificant role in peptide-mediated inhibition. The small difference in percentage inhibition by GRGDS could be easily explained by inevitable inaccuracies in counting such large numbers of colonies per lung.

Discussion

In this study we have extended our previous work that established inhibition of B16-F10 experimental metastasis by GRGDS, a peptide derived from the cell adhesion site of fibronectin, and now report on the efficacy of peptide treatment for prevention of metastatic disease and its biological characterization after administration to mice. We conclude that GRGDS is able to prevent metastasis to such an extent that recipient mice survive substantially longer than mice not receiving peptide; that GRGDS inhibition is highly specific, is not restricted to effects on tumor cell attachment, but for the most part occurs over the initial postinjection period (0–6 h); and that no deleterious or stimulatory effects on blood cell function in metastasis (platelets, NK cells, other immune cells) were detected as a consequence of GRGDS administration.

By examining the survival time for animals receiving a single coinjection of B16-F10 cells and GRGDS, we have demonstrated that the peptide is effective at preventing formation of metastases such that not only is colony formation inhibited, but also that this inhibition translates into a substantial prolongation of life-span. 100% of mice receiving 3 mg of GRGDS along with 1×10^6 or 3×10^6 B16-F10 cells were still alive > 150 d later and were therefore classified as long-term survivors. Although metastases are often present at the time of clinical presentation of the primary tumor, anti-cell adhesive peptides may be useful as an adjuvant to standard chemother-

Table III. Effect of Acetylsalicylic Acid on GRGDS-mediated Inhibition of B16-F10 Experimental Metastasis

Treatment of mice	Treatment of cells	Colony number (mean \pm SE)	Inhibition %	Colony number, median (and range)	P
None	Control	224 \pm 27.2	—	219 (110–341)	—
None	GRGDS	15.4 \pm 2.3	93	13 (11–29)	<0.001
ASA	Control	161 \pm 11.6	28	151 (120–209)	0.05
ASA	GRGDS	16.5 \pm 3.9	90	13 (7–40)	<0.001

7×10^4 B16-F10 cells were coinjected with or without 6 μmol GRGDS into groups of control mice or mice given 3.5 mM acetylsalicylic acid (ASA) in their drinking water for 3 d prior to injection. Colony formation was determined after 14 d. P values were estimated by Mann-Whitney U test. GRGDS-treated groups were compared with their respective untreated controls and the acetylsalicylic acid control was compared with the complete negative control.

Recombinant Fusion Pypeptide with Cell- and Heparin-binding Domains of Fibronectin Inhibits Liver Metastasis of L5178Y-ML25 Lymphoma Cells

Ikuro Saiki,^{1,4} Yoshihiro Matsumoto,¹ Jun Murata,¹ Takashi Makabe,¹ Junya Yoneda,¹ Harue Okuyama,² Fusao Kimizuka,³ Yukuo Ishizaki,³ Ikuonoshin Kato³ and Ichiro Azuma¹

¹Section of Chemistry and ²Section of Immunopathogenesis, Institute of Immunological Science, Hokkaido University, Kita-15, Nishi-7, Kita-ku, Sapporo 060 and ³Biotechnology Research Laboratories, Takara Shuzo Co., Ltd., Seta 3-4-1, Otsu 520-21

We have investigated the effect of recombinant polypeptides with cell-binding domain (C-274) or with heparin-binding domain (H-271) and their fusion polypeptide (CH-271) on liver metastasis of murine lymphoid tumor. The polypeptides containing heparin-binding domain, H-271 and CH-271, were able to inhibit liver metastasis when co-injected i.v. with L5178Y-ML25 T-lymphoma cells, while C-274 with cell-binding domain showed much weaker antimetastatic activity. Treatment with H-271 or CH-271 substantially prolonged the survival time of mice injected i.v. with L5178Y-ML25 cells. CH-271, containing cell- and heparin-binding domains, was more antimetastatic than H-271. The reason why CH-271 was more effective in inhibiting liver metastasis than H-271 can not be explained in terms of a difference in the stability in the circulation or in the molecular size of the polypeptide. The polypeptides used in this study did not affect the tumor cell growth or viability *in vitro*. CH-271 was found to be still active in inhibiting liver metastasis even when natural killer cells or macrophages were removed from this system. Furthermore, multiple administrations of CH-271 after tumor implantation effectively inhibited liver metastasis and enhanced the survival rate as compared with H-271, C-274 and untreated control. Thus, the fusion of H-271 with C-274 (i.e. CH-271) augments the antimetastatic property of H-271, possibly through the interaction between tumor cells and the heparin-binding domain of fibronectin.

Key words: Recombinant fibronectin fragment — Metastasis — Lymphoma cell

During the sequential steps of metastasis, metastasizing tumor cells interact with various host cells (platelets, lymphocytes or endothelial cells) and/or extracellular matrix and basement membrane components (fibronectin and laminin). Such an encounter may lead to enhancement of survival, arrest, or invasiveness of tumor cells,¹⁻³ and is therefore a fundamental event in the metastatic process.

DNA technology has allowed us to identify the primary structures of some cell adhesion proteins such as fibronectin,⁴ vitronectin,⁵ and laminin,^{6,7} and the receptors for some adhesive molecules on the cell surface.⁸ A common and characteristic Arg-Gly-Asp (RGD) core sequence in cell-binding domain of fibronectin and other related adhesion molecules has been shown to contribute to cell functions including adhesion, spreading and migration.^{9,10} Several studies have suggested that some synthetic peptides corresponding to fragments of the adhesion molecules that are present in cell matrices, basement membranes or plasma can modulate the mechanism involved in the metastasizing function of tumor cells. A proteolytic or synthetic fragment of laminin has been used to inhibit experimental metastasis.¹¹⁻¹⁴ Humphries *et al.*^{15,16} have shown that treatment of

tumor cells *ex vivo* with GRGDS peptide, which is present in the cell-binding domain of fibronectin, was able to inhibit experimental lung metastasis of murine melanoma. We have recently reported that poly(RGD), which consists of the repeated RGD sequence, inhibited experimental and spontaneous lung metastases of murine melanoma cells more effectively than RGD-containing oligopeptides, as well as showing anti cell-adhesive properties.¹⁷⁻²¹

On the other hand, McCarthy *et al.*¹⁴ have shown that the *ex vivo* pretreatment of tumor cells with a purified 33-kDa heparin-binding fragment of fibronectin, which promotes tumor cell adhesion by an RGDS-independent mechanism,²² effectively inhibited experimental pulmonary metastases of melanoma or fibrosarcoma. More recently, we demonstrated that CS1 peptide, which is present within type III homology connecting segment (IIICS) of 33-kDa heparin-binding fragment of fibronectin and promotes melanoma cell adhesion,²³ is active in inhibiting lung metastasis of murine melanoma in spontaneous and experimental metastasis models.²⁴

Recent studies showed that 33-kDa heparin-binding fragment of fibronectin promoted the adhesion of lymphocytes or lymphoid tumors as well as melanoma.²⁵⁻²⁸ Tumor lines reported to produce hepatic metastases preferentially are mostly lymphoid tumors such as RAW117-

⁴ To whom correspondence should be addressed.

H10, MDAY-D2 and L5178Y-ML.^{29,30} However, the inhibitory effect on liver metastasis of murine lymphoid tumors by peptides or fragments of fibronectin remains undefined. In the present study, we examined the effect of recombinant fibronectin fragments containing heparin- or cell-binding domains and their chimeric polypeptide on liver metastasis of murine L5178Y-ML25 lymphoid tumor.

MATERIALS AND METHODS

Animals Specific-pathogen-free CDF, mice (BALB/c × DBA/2) 8–13 weeks old, were purchased from the Shizuoka Laboratory Animal Center, Hamamatsu. Mice were maintained in the Laboratory of Animal Experiment, Institute of Immunological Science, Hokkaido University, under laminar air-flow conditions. All mice used in this study were sex-matched.

Cells Liver metastatic L5178Y-ML25 T lymphoma cells, obtained from L5178Y parent cells by *in vivo* selection for invasion,²⁹ were kindly provided by Dr. A. Okura, Banyu Pharmaceutical Co. Ltd., Tokyo. L5178Y-ML25 cells were maintained in RPMI-1640 supplemented with 7.5% fetal bovine serum (FBS) and L-glutamine.

Recombinant fibronectin fragments and other reagents We prepared three kinds of recombinant fibronectin fragments (C-274, H-271 and CH-271) by expressing human fibronectin cDNA in *E. coli*, using an expression vector pUC118N/119N first described by Maki *et al.*³¹ C-274 and H-271 correspond to cell- and heparin-binding domains of fibronectin, respectively, while another polypeptide, CH-271, is a fusion protein with both a cell- and a heparin-binding domain (Fig. 1). Two plasmids, pLF5 and pLF2435, were used as a source of cDNA.³² The

cell-binding polypeptide C-274 was expressed through a recombinant plasmid pTF7221 which had been constructed mainly from pLF5 and pUC119N. The plasmid pTF7221 was derived from pTF7121, which expresses a cell-binding polypeptide C-279 with five additional amino acids at the carboxyl-terminus of C-274. The heparin-binding polypeptide H-271 was expressed by use of a recombinant plasmid pHD101; this had been constructed from pLF2435 and pUC118N. The fusion protein CH-271 was expressed by use of a recombinant plasmid pCH-101; this had been constructed from pHD101 and pTF7121. Detailed accounts of these constructions and expressions will be given elsewhere.³³ The recombinant fragment C-274 expressed in *E. coli* was purified from the cell extract by DEAE ion exchange chromatography, followed by SP ion exchange chromatography. Fragments H-271 and CH-271 were purified by CM ion exchange chromatography, followed by affinity chromatography with heparin as a ligand. The purity of these polypeptides was verified by SDS-polyacrylamide gel electrophoresis. The amino-terminal sequence was checked with an automated peptide sequencer, model 477A (Applied Biosystems Inc., Foster City, CA). The carboxyl-terminal amino acid was also determined by use of carboxypeptidase P (Takara Shuzo Co. Ltd., Kyoto). Poly (RGD), which consists of a repeated Arg-Gly-Asp (RGD) sequence, was prepared by the synthesis of the monomer RGD peptide by the conventional method followed by polymerization with diphenylphosphoryl azide, as described previously.^{17–19} Poly (RGD) was estimated to have an approximate average molecular weight of 10,000, as assessed by viscometric measurements, SDS-polyacrylamide gel electrophoresis and gel permeation chromatography. These polypeptides were

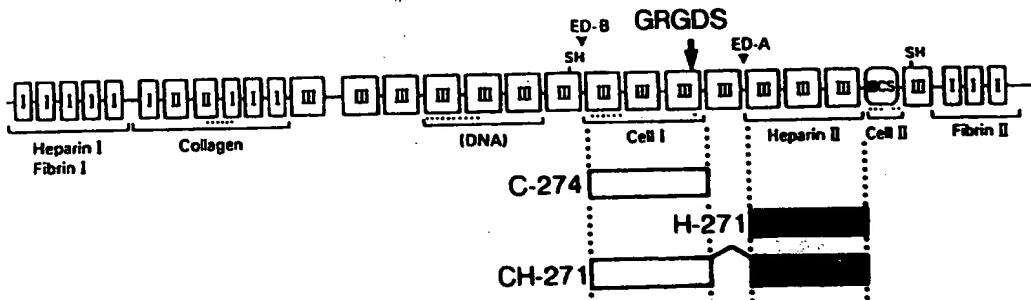


Fig. 1. Schematic diagram of recombinant fibronectin fragments. Locations of the fragments are shown by open and closed bars. The cell-binding polypeptide C-274 (Pro1239-Asp1512; the sequence is numbered according to the system of Kornblith⁴) covers the three units of type III homology at the cell-binding domain. The heparin-binding polypeptide H-271 (Ala1690-Thr1960) covers the complete region of the heparin-binding domain. The fusion polypeptide CH-271 (Pro1239-Ser1515-Met-Ala1690-Thr1960) contains both the cell- and heparin-binding domains. The boxes at the top represent the locations of the type I, II and III homology repeats. The vertical arrow indicates the GRGDS site. ED-A and ED-B indicate that extra domains arise from alternative splicing.

dissolved in Ca^{2+} - and Mg^{2+} -free phosphate-buffered saline (PBS) before use. All the reagents and media in this study were endotoxin-free ($<1.0 \text{ ng/ml}$) as determined by a colorimetric assay (Pyrodrick, Seikagaku Kogyo Co. Ltd., Tokyo).

Assay for liver metastasis of lymphoid tumors CDF₁ mice were given i.v. injection of L 5178 Y-ML 25 lymphoma (4×10^4) with or without recombinant fragments of fibronectin in PBS. Fourteen to seventeen days later, the mice were killed and the weights of liver and spleen were recorded to evaluate tumor metastasis as previously described in detail.²⁹ The survival time of the animals given i.v. injection of tumor cells with or without recombinant fibronectin fragments was also determined by allowing the animals to live until they succumbed naturally from the tumor burden. Animals were autopsied at the time of death to verify the presence of the tumor in the liver. The % survivors was calculated as a function of time.

Labeling of recombinant fibronectin polypeptides CH-271 and H-271 polypeptides were iodinated by using Bolton-Hunter reagent according to the conventional procedure. Briefly, the polypeptide (1 mg) was dissolved in 100 μl of PBS, and added to 1 mCi of Bolton-Hunter reagent (N-succinimidyl-3-(4-hydroxy-3,5-[¹²⁵I]diiodophenyl)propionate; specific activity 2000 Ci/mmol; New England Nuclear) freshly dried from a solution in benzene. After agitation of the mixture at 4°C overnight, the reaction was quenched by addition of 5 μl of 1 M glycine in borate buffer. Iodinated polypeptide was separated from by-products by gel filtration on Sephadex G-25, equilibrated and eluted with 0.05 M phosphate buffer (pH 7.5). The absorbance at 280 nm of the ¹²⁵I-labeled polypeptide thus obtained was measured with a spectrophotometer.

Procedure for study of clearance of ¹²⁵I-labeled polypeptide *in vivo* CDF₁ mice were given i.v. injections of ¹²⁵I-labeled CH-271 or H-271 polypeptide ($3.46 \times 10^6 \text{ cpm}/20 \mu\text{g}$ or $5.65 \times 10^6 \text{ cpm}/20 \mu\text{g}$, respectively) in a volume of 0.2 ml of PBS. After various times, mice were exsanguinated, and the lungs, liver, kidneys, spleen, and blood were collected and rinsed in 70% ethanol. The radioactivity in each organ was measured in a gamma counter.

Statistical analysis The statistical significance of differences between the groups was determined by applying Student's two-tailed *t* test or Mann-Whitney's U-test.

RESULTS

Effect of recombinant polypeptides on liver metastasis of lymphoid tumor We first examined the effect of the polypeptides with functional domains on liver metastasis of L 5178 Y-ML25 lymphoma cells. Polypeptides were

Table I. Effect of Recombinant Fibronectin Fragments on Liver Metastases by i.v. Injection of L5178Y-ML25 Lymphoma Cells

Co-injected i.v. with	Dose ($\mu\text{g}/\text{mouse}$)	Mean weight (g) \pm SD	
		Liver	Spleen
Untreated (PBS)		4.35 \pm 0.50	0.24 \pm 0.04
C-274	100	3.78 \pm 0.62	0.23 \pm 0.03
	250	4.14 \pm 0.84	0.22 \pm 0.03
	500	2.42 \pm 0.58**	0.21 \pm 0.05
H-271	100	2.72 \pm 0.80*	0.17 \pm 0.05
	250	2.24 \pm 0.75**	0.14 \pm 0.05*
	500	1.42 \pm 0.35**	0.11 \pm 0.02**
CH-271	50	1.83 \pm 0.92**	0.18 \pm 0.05**
	100	0.90 \pm 0.05**	0.08 \pm 0.01**
	250	0.90 \pm 0.07**	0.08 \pm 0.01**
	500	0.90 \pm 0.08**	0.08 \pm 0.02**
Poly(RGD)	500	2.72 \pm 1.04*	0.16 \pm 0.06
(Normal mice)		0.81 \pm 0.09	0.08 \pm 0

Five CDF₁ mice per group were inoculated i.v. with L5178Y-ML25 (4×10^4) with or without recombinant fibronectin fragments or poly(RGD). Mice were killed 16 days after tumor inoculation. *; $P < 0.02$. **; $P < 0.001$.

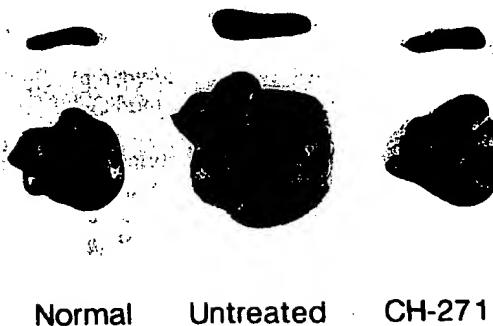


Fig. 2. Effect of CH-271 polypeptide on liver and spleen metastases of L5178Y-ML25 lymphoma cells. CDF₁ mice were given i.v. tumor cells (4×10^4) admixed with or without 100 μg of polypeptide. Mice were killed 14 days after tumor inoculation.

co-injected i.v. with L5178Y-ML25 lymphoma cells into CDF₁ mice (Table I). The polypeptides containing the heparin-binding domain, H-271 and CH-271, significantly inhibited liver and spleen metastases of L5178Y-ML25 lymphoma cells at concentrations ranging from 50 to 500 μg per mouse. CH-271, a fusion polypeptide

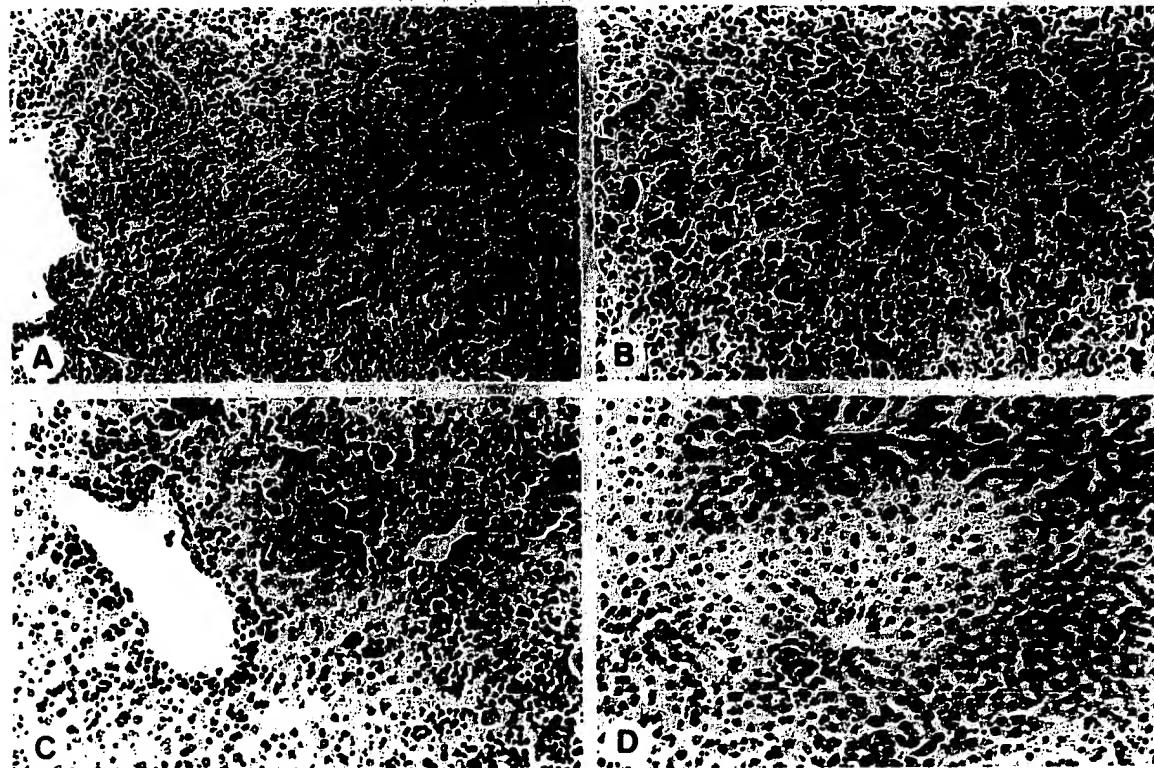


Fig. 3. Liver metastasis of L5178Y-ML25 lymphoma cells. A, Control (untreated). All the liver cells are replaced by tumor metastases. B, C-274 (500 µg)-treated. A small number of liver cells remains among proliferating tumor cells. C, H-271 (500 µg)-treated. Diffuse and focal tumor metastases are present around the vessels and in the sinusoids. D, CH-271 (100 µg)-treated. Only a few mononuclear foci, but no tumor cells scattered in the liver tissue. Hematoxylin-eosin staining $\times 160$.

with both cell- and heparin-binding domains, was more active than H-271 in inhibiting tumor metastasis and decreased the liver and spleen weights to the normal level (Table I and Fig. 2). In contrast, C-274 with the cell-binding domain and poly(RGD), which consists of repeated RGD sequences (in the cell-binding domain of fibronectin), achieved less reduction of liver and spleen weights at any dose than did H-271 and CH-271. Histological analysis revealed that the enlarged liver in the control (untreated) group exhibited diffuse infiltration of tumor cells, and no liver cells were seen (Fig. 3A). The C-274-treated group showed many size variations of foci of tumor metastasis from large to small. A few liver cells remained among the metastatic foci (Fig. 3B). In the H-271-treated group (Fig. 3C), tumor cells proliferated around the vessels and in the sinusoids, but they were far smaller and fewer than those of the C-274-treated group. No metastasis was found in the CH-271-treated group but there were quite a few small foci of mononuclear cells

(Fig. 3D). The survival rate of mice given i.v. injection of L5178Y-ML25 lymphoma cells admixed with the polypeptides was also determined in the experimental liver metastasis model (Fig. 4). In this experiment, 50% of untreated mice succumbed to the tumor burden within 16 days of tumor cell inoculation. Similar survival rates were observed in the group of mice which received L5178Y-ML25 cells admixed with C-274 polypeptide. The group that received H-271 showed an enhanced survival rate, but 6 out of 10 mice had succumbed within 50 days of the tumor cell inoculation. All the mice given CH-271 were still alive at 50 days following tumor cell injection. Table II shows that the incubation of tumor cells with various concentrations of polypeptides did not affect the incorporation of [³H]thymidine into tumor cells or the cell viability determined by the trypan blue dye exclusion test. The peptidic anticancer drug neocarcinostatin (10 µg/ml), used as a positive control, potently inhibited the cell growth *in vitro*. This result

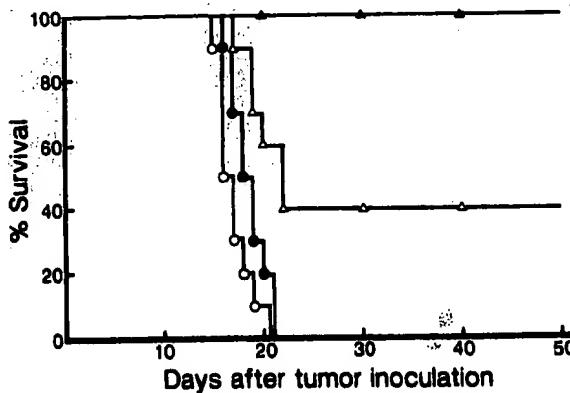


Fig. 4. Effect of recombinant fragments of fibronectin on the survival of CDF₁ mice co-injected i.v. with L5178Y-ML25 lymphoma cells. Mice were injected i.v. with L5178Y-ML25 (4×10^6) together with PBS (○), or 250 μ g of C-274 (●), H-271 (Δ) or CH-271 (\blacktriangle) and animal survival was monitored as a function of time.

indicates that the polypeptides were not cytotoxic and did not inhibit cell growth.

Effect of anti-asialo GM1 serum or 2-chloroadenosine on CH-271-mediated inhibition of tumor metastasis Since NK cells or macrophages in the circulation play an important role in the inhibition of tumor metastasis,^{34,35} we investigated whether or not the action of CH-271 polypeptide requires NK or macrophages to induce the inhibition of tumor metastasis. Anti-asialo GM1 serum can selectively eliminate NK cells³⁶ and 2-chloroadenosine is a macrophage-toxic substance.³⁷ Table III shows that pretreatment of mice with anti-asialo GM1 serum is likely to enhance slightly the frequency of liver metastasis as compared with the frequency found among untreated mice but pretreatment with 2-chloroadenosine does not enhance it significantly. The co-injection with CH-271 led to a significant reduction of liver metastasis in both untreated and treated mice.

Retention of 125 I-labeled polypeptide in the circulation We next examined the behavior of H-271 or CH-271 polypeptide in the circulation and various organs. Figure 5 indicates that there is no discernible difference between H-271- and CH-271-injected mice in the clearance of labeled polypeptide in the blood and organs after the injection. The clearance of labeled polypeptide from the circulation was biphasic and rapid during the early phase after the injection. A very rapid initial decrease in polypeptide was obtained within 5 min after the injection, implying that the initial decrease would probably be due to rapid equilibration or a dilution effect in the body

Table II. Effect of Recombinant Fibronectin Fragments on the Growth and Viability of L5178Y-ML25 Lymphoma Cells

Treatment	Concentration (μ g/ml)	Incorporation of [3 H]thymidine into the cells (cpm)	Viability (%)
Untreated (medium)		227111 \pm 10635	98
C-274	0.1	183221 \pm 24430	
	1	185022 \pm 1171	
	10	183331 \pm 36593	
	100	188147 \pm 6650	98
H-271	0.1	211488 \pm 868	
	1	200610 \pm 15067	
	10	215323 \pm 9250	
	100	240020 \pm 5063	100
CH-271	0.1	193450 \pm 4204	
	1	218670 \pm 9688	
	10	189178 \pm 10883	
	100	193912 \pm 26188	97
Neocarcinostatin	10	1409 \pm 457	2

L5178Y-ML25 cells (5×10^3) were incubated with medium, recombinant fibronectin fragments or neocarcinostatin for 3 days at 37°C. The culture was pulsed with 0.5 μ Ci of [3 H]-thymidine for the last 4 h before termination. Cell viability was assessed by the trypan blue dye exclusion method 3 days after the co-incubation.

Table III. Effect of Anti-asialoGM1 IgG or 2-Chloroadenosine on CH-271-mediated Inhibition of Liver Metastases of L5178Y-ML25 Lymphoma Cells

Treatment of mice	CH-271	Mean weight (g) \pm SD	
		Liver	Spleen
None	—	2.99 \pm 0.53	0.19 \pm 0.02
	+	0.97 \pm 0.09*	0.08 \pm 0.02*
Rabbit anti-asialoGM1 IgG, 20 μ l, i.v.	—	3.72 \pm 0.36	0.26 \pm 0.05
	+	0.96 \pm 0.06*	0.10 \pm 0.02*
2-chloroadenosine, 50 μ g, i.v.	—	3.14 \pm 0.62	0.19 \pm 0.04
	+	1.40 \pm 0.25*	0.10 \pm 0.01
(Normal mice)		0.98 \pm 0.07	0.08 \pm 0

L5178Y-ML25 cells (4×10^6) were injected i.v. with or without 100 μ g of CH-271 into groups of control CDF₁ mice or mice pretreated 24 h earlier with antibody or 2-chloroadenosine. Mice were killed 14 days after tumor inoculation.

*; $P < 0.001$.

fluid. After the initial equilibration, the clearance of labeled polypeptide was rapid up to 1 h and thereafter the labeled polypeptide was gradually cleared. The half-lives

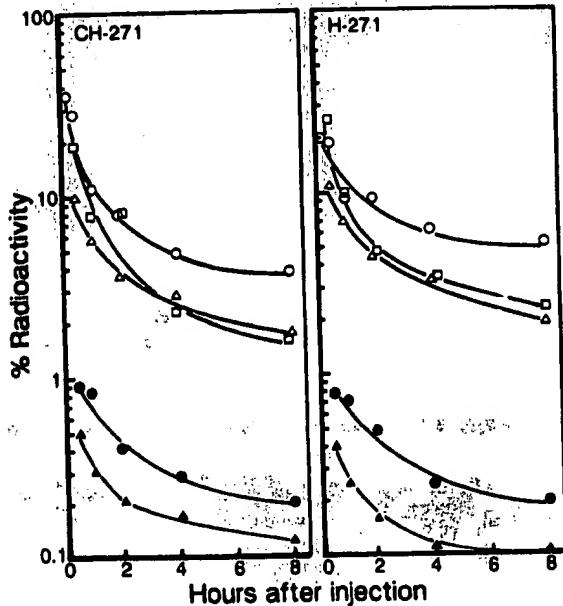


Fig. 5. Clearance of ^{125}I -labeled polypeptides from the circulation in CDF₁ mice. The levels of radioactivity in blood; (○), lungs; (●), liver; (△), spleen; (▲) and kidneys; (□) were determined as described in "Materials and Methods." The first samples were collected after 5 min. Each point represents the mean of a group of three mice.

of the polypeptide during the early and late phases were approximately 15 min and 3 h.

Effect of administration timing of CH-271 fusion polypeptide on liver metastasis of L5178Y-ML25 lymphoma cells. We next examined the effect of administration protocol of the CH-271 polypeptide on liver metastasis of L5178Y-ML25 cells. CH-271 at a dose of 100 μg significantly suppressed the increase of liver and spleen weights upon co-injection (admixing) with L5178Y-ML25 cells (Table IV). In addition, i.v. administration of CH-271 polypeptide 1 h before or after tumor injection significantly inhibited the liver metastasis. Pretreatment of L5178Y-ML25 cells with 100 μg of CH-271 for 30 min also achieved the inhibition of metastasis.

Therapeutic effect of multiple administrations of polypeptides on liver metastasis of L5178Y-ML25 lymphoma cells. Multiple treatments with polypeptides at the dose of 250 μg were performed daily for 7 days after tumor inoculation. Table V shows that the i.v. administrations of CH-271 significantly suppressed the increase of liver and spleen weights. Multiple treatments with H-271 or C-274 also showed significant suppression of the increase of liver and spleen weights, but were less effective than CH-271 fusion polypeptide. The survival rate of mice given multiple i.v. administrations of polypeptides for 7 days after tumor inoculation was also determined (Fig. 6). In this experiment, 50% of untreated control mice succumbed to the tumor burden within 20 days of the injec-

Table IV. Effect of Injection Timing of CH-271 Polypeptide on Liver Metastases by i.v.-Injected L5178Y-ML25 Lymphoma Cells

Administered i.v. with	Timing	Dose ($\mu\text{g}/\text{mouse}$)	Mean weight (g) \pm SD	
			Liver	Spleen
Expt. I				
Untreated (PBS)				
CH-271	Admix	100	3.22 ± 0.86	0.19 ± 0.03
	Separate ^a			
	1 h before	100	$1.01 \pm 0.08^{**}$	$0.10 \pm 0.01^{**}$
	1 h after	100	$1.67 \pm 0.21^{**}$	$0.11 \pm 0.01^{**}$
(Normal mice)			$1.71 \pm 0.51^{*}$	0.14 ± 0.02
			0.95 ± 0.04	0.09 ± 0.01
Expt. II				
Untreated (PBS)				
CH-271	Admix	100	3.36 ± 0.42	0.18 ± 0
	Pretreatment ^b	100	$0.91 \pm 0.10^{**}$	$0.10 \pm 0.01^{**}$
			$0.88 \pm 0.08^{**}$	$0.08 \pm 0.01^{**}$

Five CDF₁ mice per group were inoculated i.v. with L5178Y-ML25 (4×10^6) cells with or without CH-271. Mice were killed 14 days after tumor inoculation.

a) CH-271 was administered i.v. at the indicated time points before or after the i.v. injection of L5178Y-ML25 cells.

b) The cells were incubated with CH-271 for 30 min and then washed three times before injection.

*; $P < 0.01$. **; $P < 0.001$.

Table V. Therapeutic Effect of Recombinant Fibronectin Fragments on Liver Metastasis by L5178Y-ML25 Lymphoma Cells

Administered with	Dose ($\mu\text{g}/\text{mouse}$)	Mean weight (g) \pm SD	
		Liver	Spleen
—	—	5.16 \pm 0.42	0.26 \pm 0.02
CH-271	250 \times 7	1.45 \pm 0.23*	0.14 \pm 0.01*
H-271	250 \times 7	3.56 \pm 0.37*	0.20 \pm 0.02
C-274	250 \times 7	3.25 \pm 0.64*	0.18 \pm 0.06
(Normal)		1.12 \pm 0.11	0.08 \pm 0

Five CDF₁ mice were implanted i.v. with 4×10^4 L5178Y-ML25 lymphoma cells, and administered i.v. with or without 250 μg of the polypeptides for 7 days after tumor inoculation. The treatments with polypeptides were started 1 day after tumor inoculation. Mice were killed 13 days after tumor inoculation. *, $P < 0.001$.

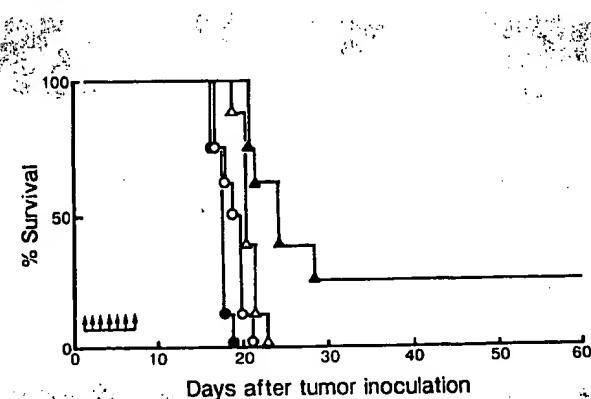


Fig. 6. Effect of multiple administrations of polypeptides on the survival of CDF₁ mice injected with L5178Y-ML25 lymphoma cells. Mice were administered i.v. with PBS (○), C-274 (●), H-271 (△) or CH-271 (▲) 250 μg per day for 7 days (—) after i.v. injection of tumor cells. The treatments with polypeptides were started 1 day after tumor inoculation. Animal survival was monitored as a function of time. $P < 0.01$; CH-271 vs. untreated control.

tion. Similar survival rates were observed in the group of mice which received C-274 or H-271 polypeptide. The group that received CH-271 showed a significantly enhanced survival rate ($P < 0.01$ by Mann-Whitney's U test), but 6 out of 8 mice had succumbed within 30 days of the tumor inoculation.

DISCUSSION

We have attempted to elucidate the mechanisms involved in cell functions such as adhesion and motility during the metastatic process. Tumor cell adhesion to components of the extracellular matrix, in particular

fibronectin, is an important aspect of several steps of metastasis.¹⁻³ Previous studies have utilized proteolytic fragments or synthetic peptides of laminin or fibronectin to inhibit experimental metastasis of tumor cells in mice.¹¹⁻¹⁶ We also demonstrated that synthetic peptides derived from fibronectin such as poly(RGD), CS1 or recombinant fragments with functional domains can be used to inhibit experimental and spontaneous lung metastases of murine melanoma, tumor cell adhesion to the extracellular matrix, the penetration of tumor cells through reconstituted basement membrane *in vitro* and tumor-induced angiogenesis in syngeneic mice.^{17-21, 24}

To extend our previous observation on the inhibition of tumor metastasis by synthetic and recombinant polypeptides, we have examined the behavior of recombinant polypeptides with the cell- or heparin-binding domain or the fusion polypeptide on liver metastasis of lymphoid tumor *in vivo*. Co-injection of L5178Y-ML25 lymphoma cells with the polypeptides containing heparin-binding domain (H-271 and CH-271) resulted in marked suppression of the increase of liver and spleen weights, but C-274 with the cell-binding domain and poly(RGD) inhibited liver metastasis only at the high dose (500 $\mu\text{g}/\text{mouse}$) (Table I and Figs. 2 and 3). H-271 or CH-271 significantly enhanced the survival rate as compared with untreated control or C-274 (Fig. 4). These results clearly indicate that the heparin-binding domain of fibronectin (H-271 and CH-271) dramatically inhibited liver metastasis of L5178Y-ML25 lymphoma cells. Furthermore, significant inhibition of liver metastasis was observed when CH-271 was injected i.v. 1 h before or after the injection of L5178Y-ML25 cells as well as when a mixture of CH-271 and tumor cells was co-injected. We therefore concluded that the cells do not require a prolonged incubation with polypeptide. The polypeptides used, however, did not exhibit direct cytotoxicity against tumor cells, nor did they affect cell growth (Table II). This suggests that the inhibitory effects of the polypeptides on tumor metastasis cannot be simply explained by direct cytotoxicity toward tumor cells.

Tumor cells in the circulation interact with host cells such as lymphocytes, natural killer (NK) cells and monocytes, which are believed to be particularly important in killing tumor cells.^{34, 35} CH-271 significantly inhibited liver metastasis in mice pretreated with anti-asialo GM1 serum or 2-chloroadenosine as well as untreated mice (Table III). Since CH-271 was still active when NK cells and macrophages were removed from our system, its inhibitory mechanism is unlikely to be directly related to the stimulation and activation of these cells. We recently observed that the co-injection of tumor cells with polypeptides containing the heparin-binding domain led to a significantly reduced arrest of tumor cells in lung and liver over 8 h after injection, and we showed that these

polypeptides were able to inhibit tumor cell adhesion to substrates coated with reconstituted basement membrane component, Matrigel.³⁸⁾ The adhesive interaction of tumor cells with CH-271 was inhibited by the addition of heparin or monoclonal antibodies against the heparin-binding domain of fibronectin.³⁸⁾ Thus, the inhibitory effect on liver metastasis by CH-271 may be attributable to the interaction between the tumor cell surface and the heparin-binding domain rather than the cell-binding domain in the polypeptide molecule. In support of this notion, the interaction between heparin-like molecules on the cell surface and the heparin-binding domain in fibronectin could modulate haptotactic migration of metastatic melanoma to fibronectin-substrate.³⁹⁾

Among these polypeptides, CH-271 fusion polypeptide inhibited liver metastasis of L5178Y-ML25 lymphoma cells more effectively than H-271 on a weight basis (Table I). The clearance rate of ¹²⁵I-labeled CH-271 from the circulation after the i.v. injection is similar to that of labeled H-271, and the half-life of both polypeptides during the late phase (after 1 h) is approximately 3 h (Fig. 5). These results indicate that the inhibition of metastasis of L5178Y-ML25 cells by CH-271 or H-271 may not depend on a difference in the stability of the polypeptides in the circulation after injection, or in the molecular size. Since we observed that a mixture of H-271 and C-274, or C-274 alone was much less active in inhibiting tumor metastasis than CH-271 fusion polypeptide at a similar molar ratio (Table I and reference 38), the fusion of H-271 with C-274 may facilitate the inhibitory effect on tumor metastasis, or possibly the interaction between tumor cells and the heparin-binding domain (perhaps by altering the binding affinity). Further study is needed.

Multiple administrations of CH-271 after tumor implantation significantly inhibited liver metastasis of

L5178Y-ML25 cells and enhanced the survival rate as compared with the untreated control, whereas multiple treatments with H-271 or C-274 did not (Table V and Fig. 6). The exact mechanism responsible for the inhibition of liver metastasis by CH-271 is not known yet, but may be more complex than a simple interference with tumor cell adhesion to the extracellular matrix. Further study will be needed to determine the inhibitory effect on the invasion and enzymatic degradation of the extracellular matrix by L5178Y-ML25 cells and so on.

Finally, the present study has demonstrated that recombinant fusion polypeptide of fibronectin (CH-271) inhibited liver metastasis of L5178Y-ML25 T-lymphoma cells, possibly through a mechanism mediated by the heparin-binding domain, and enhanced the survival rate. Our previous study showed that C-274 (containing RGD sequence) or CS1 peptide could inhibit pulmonary metastasis of murine melanoma by an RGD-dependent or -independent mechanism, respectively.²⁴⁾ More recently, we observed that CH-271 also inhibited liver metastasis of RAW117-H10 B-lymphoma cells (data not shown). Thus, since the fusion polypeptide of fibronectin showed no short-term toxicity to the host, it may be potentially useful in the prevention of cancer metastasis.

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